Developing Manduca sexta as a model for microbiome research

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Developing *Manduca sexta* as a Model for Microbiome Research

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Table of Contents

List of Figures

List of Tables

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Abstract

The microbiomes of complex animals play important roles in the health of their hosts, and microbiome research is an area of intense activity. However, while vertebrate models such as mice are the model of choice for such studies, the complexity of the gut microbiome of these rodents, and the cost and ethical implications of their use are barriers to their use. The aim of this thesis is to develop a simple model for studying the host-microbiome interactions, using the in-house model insect *Manduca sexta* (tobacco hornworm).

The growth of *M. sexta* is characterized by five larval instars, with moulting through each of the instars occurring, with the first instar larva hatching after 1-3 days. During the fifth instar, the larva undergoes the most changes before pupation and as such, most studies involving research of the gut microbiome is usually performed at this stage in the growth of these larvae. In this study, the resident gut microbiome of *M. sexta* larvae was characterized using both culture-based and culture-free methods to carry out the taxonomic identification of the resident gut microbiota of the larvae.

16S rRNA gene Sanger sequencing revealed the identification of bacterial diversity was recovered from different diet (with or without tetracycline supplementation of standard colony food) larvae groups, agars and rearing conditions using direct culture-dependent method. The occurrence and predominance of isolates were spore-forming gram-positive bacteria belonging to genera *Bacillus, Viridibacillus, Pseudomonas, Staphylococcus, Lysinibacillus, Oceanobacillus, Lactobacillus* and other related bacterial species. Interestingly, the percentage of 16S rRNA gene sequence similarity of all isolates was above 99% except for isolate MS7

that showed 97.69% similarity with *O. massilliensis* with differences in 17bp indicating that the isolate was a potential new bacterium species. The use of enrichment as an alternative method for the identification of the resident gut microbiota in the larvae did not allow a wider microbiome profile to be identified, while the culture-free method permitted a higher number of taxonomic identifications of bacterial species but the very low concentration of gDNA in these samples made them sensitive to contamination by environmental DNA. This technical difficulty might be attributed to the low depth coverage of some sequence runs of some isolates using Illumina Miseq platform 16S v4 rRNA gene sequencing. Despite using these methods, the newly discovered isolate *O. massilienesis* was not among isolates that were isolated from the larval gut samples. Interestingly, *Firmicutes* bacteria were the major predominant phylum observed in all larval bacterial gut across all samples.

A protocol to rear bacteria-free *M. sexta* was developed. However, the effect of depleting or reintroducing the gut microbiome (colony foodborne bacteria and environment) during pre-maturation (day 8) of these larvae revealed a novel and critical role for gut bacteria in the growth and development of these insects. This is contrary to previous studies but highlights a key difference in the generation of bacteria-free larvae, rather than using antibiotics to suppress bacterial growth that was used in previous studies. This project identifies *M. sexta* as a model in which the role of gut bacteria on host growth and development can be studied.

List of Abbreviations

16S MTP- 16S Microbiome Taxonomic Profile.

16S rRNA gene- A marker gene used for bacteria and Archaea identification.

AMPs- Antimicrobial Peptides.

Antibiotics cocktail- A mix of a wide-spectrum of antibiotics.

BA- Blood agar.

BF- Bacteria-free *M. sexta* eggs/larvae.

BHI- Brain Heart Infusion broth/agar medium.

Blank-control- Sample-free reagents/kit.

BLASTN- Basic Alignment Search Tool for nucleotides or proteins sequences.

CFU- Cell Forming Unit.

de MRS- de Man and Rogosa and Sharp agar/broth medium.

DEFRA- General licences for wild-life breading or management by Gov.uk.

DGGE- Denaturing Gradient Gel Electrophoresis.

DNA- Deoxyribonucleic acid.

DNA-DNA Hybridization The degree of sequence similarity between mixed DNAs.

EzBiocloud- An up to date online manually curated database server for academic user to conduct genome analyses.

GE- Gel Electrophoresis.

GF- Germ-free animal models.

HF%- Hatchling frequency percentage of *M. sexta* eggs.

IBD- Immune bowl disease or syndrome.

Illumina Hiseq or Miseq platform- second generation of the high throughput sequencing technology.

ITS- Nuclear ribosomal Internal Transcribed Spacer.

LLP-1- Lysozyme Like-protein 1.

LPS- Lipo-Polysaccharides of outer membrane of gram-negative bacterial cell.

LSU- Large subunit of 18S (eukaryotes) or 16S (procaryotes) rRNA gene sequence.

MacConkey- MacConkey agar medium.

MALTDI-TOF Matrix-Assisted Laser-Desorption-Ionization Time of Fly.

Microarrays- Molecular tools used for detection of e.g., gene expression via spots on a solid surface.

Mock Microbial community- A defined mixed culture (e.g., ZymoBIOMICS™ mock microbial community).

MP- Mortar and Pastel tool.

NA- Nutrient agar medium.

NCBI- National Centre for Biotechnology Information.

NGST- Next Generation Sequencing Technology.

OD_{600nm}- Optical density at 600nm wavelength.

OGRI- Overall genome related index or values.

OTUs- Operational Taxonomic Units.

PBS- Phosphate buffer saline.

PCR- Polymerase chain reaction.

pH- Quantitative of acidity or basicity of a solution (power of $[H^+]$ ion concentration).

QIIME 2- Bioinformatics tools or package used for 16S taxonomic analyses.

QPCR- Quantitative PCR.

RAPD-PCR Random and Amplification Polymorphic DNA-based PCR.

RNA- Ribonucleic acid.

RT-PCR Reverse Transcription based PCR.

SA-food- Sterile antibiotic supplemented food.

SA-free food- Sterile antibiotic-free food.

SIP- Stable Isotope Probing.

SNPs- Single Nucleotide Polymorphisms.

SSCP-PCR Single Stranded DNA Conformation Polymorphism.

SSU- Small subunit of 18S (eukaryotes) or 16S (prokaryotes) rRNA gene sequence.

TS- Tryptone soy agar/broth medium.

UV- Ultra violate rays.

V1-9 Hypervariable regions of the 16S rRNA gene.

WGS- Whole Genome Sequencing technology.

1.Chapter 1 INTRODUCTION

1.1 Phylogeny of Lepidoptera

Moths and butterflies are composed of a diverse number of insect orders with more than 157,000 species that have been identified which can be grouped into 43 superfamilies and 133 families (Triant, Cinel and Kawahara, 2018). The majority of Lepidoptera are grouped in the clade which is composed of 29 superfamilies. Figure 1.1 shows the relationships among the major superfamilies and the number of assembled genomes in the phylogeny tree of Lepidoptera (Triant, Cinel and Kawahara, 2018). Moths and butterflies have been utilized as model organisms for a diverse number of research procedures in the field of agriculture as well as understanding the developmental processes in vertebrates and the role of the microbiome in modulating the immune system and metabolism of the host organism (Triant, Cinel and Kawahara, 2018). Next generation molecular sequencing technologies have permitted the sequencing of the genomes of Lepidoptera such as the domesticated silkworm (*Bombyx mori*) (Mita *et al.*, 2004). The genomes of Lepidoptera range from approximately 246-809MB with a lesser degree of complexity than the genome of higher eukaryotes (Gouin *et al.*, 2017). To date, 21 gene assemblies have been annotated that encompass 21 species in 13 families while about 29 assemblies lack a functional annotation for 27 species in 4 families (Talla *et al.*, 2017). *Manduca sexta* (tobacco hornworm) is a common pest species from the lepidoptera order, *Sphingidae* family and the *Bombycoldea* superfamily. They are pests for plants such as tobacco and tomato (Brinkmann, Martens and Tebbe, 2008). The larval form of *Manduca sexta* is most commonly encountered and this species has been extensively used for research studies in

areas such as insect development, genetics and behaviour (Singh *et al.*, 20014), partly due to the relative ease with which *Manduca sexta* can be successfully reared on an artificial diet under laboratory conditions. Indeed, this insect has been utilized as a model organism to study the role of food in the acquisition or changes of the resident gut microbiome in insects (Woods, 1999; Brinkmann, Martens and Tebbe, 2008).

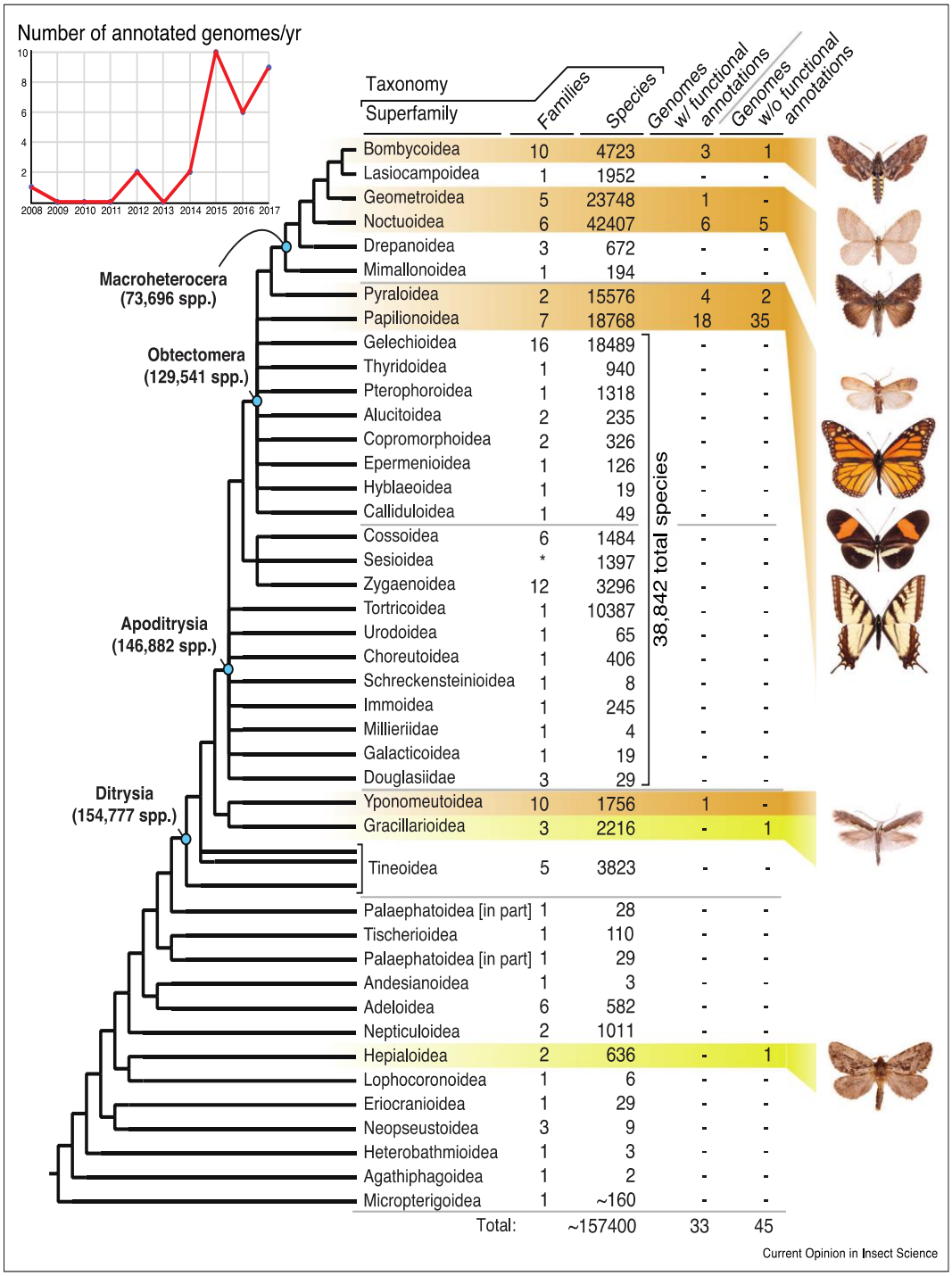


Figure 1.1: Phylogeny of Lepidoptera showing the major relationships between the superfamilies and the number of assembled genomes (Triant, Cinel and Kawahara, 2018).

1.2 Microbiome term and concept

The term “Biome” was first introduced by Clement and Shelford in 1939 who used it to describe the relationship between the plant and animal community of a given environment (Ozburn, 1940). Biome refers to biotic and abiotic entities of a specific niche, (Tipton, Darcy and Hynson, 2019). However, the concept of this term has gradually evolved among ecologists and later was used to describe the microorganism population of a host referred to as the micro-biome. The microbiome indeed plays important roles in the various metabolic activities occurring in the host (Bordenstein and Theis, 2015; Tipton, Darcy and Hynson, 2019). The development of the microbiome depends significantly on environmental factors and the host characteristics, which are variable according to the type of host e.g., plant, animal and humans. Microbiomes comprise mostly of bacteria, but also protists, archaea, fungi, and viruses can be isolated. The biological system in which the host and microorganisms develop in a symbiotic way is referred to as the holobiont. Microorganisms evolving in a holobiont can be transient or stable. Knowing the identity of the microorganisms present in a holobiont is essential in order to understand the on-going interactions, the functioning of the system as well as the beneficial or adverse outcomes of the principal metabolic activities. In such a system, the host, and the microbiome exist in a state of a balanced interdependence with the host providing an ecological niche for the microbial flora. The microbiome, in turn, helps in the normal host physiology and e.g., prevention of illnesses. It is important to understand the different ways by which microbiomes contribute to the existence of the host (Surana and Kasper, 2014).

Microbiome research focuses on three key aspects; the first aspect deals with the identification and study of microbiomes in a healthy host, the second addresses the role of the microbiome in normal host physiology and responses to the environment. The third aspect includes the study of the implication of the microbiome in the alteration of the host behaviour in any manner (Biron *et al.,* 2015). Another interesting aspect of the study of microbiomes is related to the release of information pertaining to phylogeny and evolution. With the regards to the determination of the identity and roles of the microbial communities of various hosts, there are numerous methods that can be used. Moreover, screening has become more feasible with advancements in molecular biology techniques (e.g., sequencing of specific genes or the whole genome) that allow the generation of more reliable results. For example, high-throughput sequencing has allowed an easier and more rapid determination of the essential roles played by the microbiome in the host physiology and evolution. The standard gut microbiome of humans was considered to be an inactive entity and emphasis was given more on the metabolic activities occurring at cell and gene level. The advent of next generation sequencing technology (NGS) or high-throughput sequencing, however, has made it possible to gain a better understanding into the metagenomics of the human microbiome in health and diseases (Blaser, 2013; Malla *et al.,* 2019).

1.3 Microbiomes and host physiology

Microbiomes have definite roles in various aspects of the host biology. They contribute to numerous physiological activities of the host such as the digestion and absorption of nutrients as well as the establishment and proper functioning of the host immune system.

For example, bacteria in the human colon are able to digest resistant starch and other polysaccharides that are difficult to digest leading to the release of e.g., short chain fatty acids such as acetate, propionate, and butyrate. These compounds are essential for normal bowel function and prevention of diseases. The role of butyrate has been associated with the prevention of illnesses due to the fact that it maintains normal colonocyte production (Topping and Clifton, 2001). Ever since the discovery that germ-free mice (GM), which lacked the normal microflora, had poor, underdeveloped immune systems, the idea that the normal microflora has a major role in host immunity was established. These mice had a lower number of lymphocytes than normal, a phenotype that was rescued by the administration of the normal flora. The same phenotype as that of GM mice was observed in antibiotic-treated mouse models. These results indicate the role of the microbiome in supporting efficient immunity. It is also crucial to note that microbiomes are specific to a host. Mice that were given the microbiota of humans or rats failed to establish a proper immune system. This result suggests a co-evolution of the host and the microbiome (Round and Mazmanian, 2009).

A specific bacterial species that has been proven to be beneficial to their hosts include *Bacteroides fragilis*. It is a commensal bacterium of humans that produces polysaccharide A, a compound that can correct T cell deficiencies in mice (McFall-Ngai *et al.*, 2013). *B. fragilis* has also been reported to have a protective and therapeutic role in the prevention and treatment of multiple sclerosis and colitis due to its ability to produce polysaccharide A (Mazmanian *et al*, 2005; Wang *et al*, 2006; Surana and Kasper, 2014). Other beneficial bacteria include those belonging to the genus *Clostridium* and the order *Bacteriodales* that are able to induce the production of T-regulatory cells (Tregs) in mouse

colons and provide protection against colitis and allergy. The perturbation of the microbiome and a shift in the balance of the host-microbiome composition is associated with a variety of diseases. Inflammatory bowel disease (IBD) is a chronic illness that has been associated with changes in the host microbiome composition. The progress of the disease has been linked with changes in host-microbe interactions that result from differences in the structure and function of the microbiome. It has been proven that the character of the host microbiome changes with the progress of the disease and this change determines the outcome of the disease (Dalal and Chang, 2014; Sharma and Shukla, 2016). Microbiomes have been linked to other diseases, cancer being an example. *B. fragilis* plays an important role in host immunity, but one specific strain of the species can produce an enterotoxigenic compound (metalloprotease toxin) causing a type of colitis that encourages tumour formation in the colon (Sears *et al.*, 2014).

1.4 Insect microbiomes.

Studies of insects' microbiomes have been of great importance, since these microorganisms are essential for physiology, metabolism and immune responses. The gut of insects houses many non-pathogenic microorganisms that contribute to the well-being of the organism, and it has been concluded that they participate in the sustenance and development of the immune system of lepidoptera (Tang *et al.*, 2012; Shao., *et al.*, 2017).

The nutritional effects of bacteria in Lepidoptera are of vital importance, several studies focus on the importance and binding relationships between microbes and Lepidoptera. The transient and facultative microbial communities that reside in the

intestinal tract of organisms, which could influence the variability of the host's diet are probably the least studied aspects (Lundgren and Lehman, 2010; Voirol et al., 2018).

1.4.1 Microbiomes and diet

The gut microbiome varies according to factors such as the diet, developmental stage, habitat and host. In the particular case of the diet, it is well known that this factor controls the microbial diversity. For mammals, the diversity of gut microbiota is diet-specific, from carnivores to omnivores and herbivores (Ley *et al.*, 2008). In insects such as termites and aphids, members of the intestinal microbiome digest essential elements, which are otherwise inaccessible to the host (e.g., detritus, phloem, sap, wood, and xylem) (Moran *et al.*, 2008; Tartar *et al.*, 2009; Engel *et al.*, 2012; Voirol et al., 2018). A summary of the results is shown in table 1.1.

Table 1.1: Modes of transmission, the composition and the functions of the gut microbiome of representative insects.

Insect host species	**Transmission routes**	**Number of major spp.**	**Example taxa**	**Consistency among hosts**	**Host food**	**Proposed role in hosts**	**References**
Plastaspid bug: *Megacopta punctatissima*	Maternal (egg capture)	1	*Ishikawaella capsulatus (Proteobacteria)*	Uniform	Plant sap	Nutrient supply	(Fukatsu and Hosokawa, 2002), (Hosokawa *et al.*, 2006)
Alydid bug: *Riptortus clavatus*	Environment	1	*Burkholderia sp. (Proteobacterium)*	Uniform	Plant sap	Nutritional and degradation of toxins	(Kikuchi, Hosokawa and Fukatsu, 2007; Kikuchi *et al.*, 2012)
Reed beetle: *Macroplea sp.*	Maternal egg smearing	1	*Macropleicola spp. (Proteobacterium)*	Uniform	Plant cells	Production of cocoon material	(Kölsch, Matz-Grund and Pedersen, 2009; Kölsch and Pedersen, 2010)
Rhodnius prolixus	Maternal egg smearing	1	*Rhodococcus rhodnii (Actinobacterium)*	Uniform	Blood	Nutrient supply	(Beard, Cordon-Rosales and Durvasula, 2002; Eichler and Schaub, 2002).
Honey and bumble bee: *Apis spp. Bombus spp.*	Social transmission	6-9	*Snodgrassella alvi,Gilliamella apicola, Lactobacillus spp.*	Uniform	Pollen and nectar	Digestion and protection against parasites	(Koch and Schmid-Hempel, 2011; Martinson *et al.*, 2011; Engel and Moran, 2013)

Lower termites: *Reticulitermes speratus*	Social transmission	>300	*Flagellates, Bacteroidetes, Spirochetes, Proteobacteria, Firmicutes*	Uniform	Dry wood	Nutrient supply, N_2 recycling, fixation, cellulose digestion, fermentation	(Hongoh *et al.*, 2005, 2008; Nakajima *et al.*, 2005; Desai and Brune, 2012)
Higher termites: *Nasutitermes species*	Social transmission	>300	*Spirochetes, Fibrobacteres, Bacteroidetes, Firmicutes, Acidobacteria, Proteobacteria, TG3.*	Uniform	Detritus	Nutrient supply, N_2 recycling, fixation, cellulose digestion, fermentation	(Warnecke *et al.*, 2007; Köhler *et al.*, 2012)
Grasshopper: *Schistocerca gregaria*	Acquisition from food	<12	*Enterococcus, Serratia, Klebsiella, Acinetobacter*	Variable	Plant leaves	Produce components of aggregation pheromones	(Dillon *et al.*, 2008, 2010)
Fruit fly: *Drosophila melanogaster*	Acquisition from food	<8	*Lactobacillus spp., Acetobacteraceae, Orbaceae*	Variable	Decaying fruit	Prime immune system, mating preferences and metabolism	(Broderick and Lemaitre, 2012)
Gypsy moth caterpillar: *Lymantria dispar*	Acquisition from food	<8	*Pseudomonas, Enterobacter, Pantoea, Serratia, Staphylococcus, Bacillus*	Variable	Plant leaves	Unknown. May increase susceptibility to toxins through the regulation of mid gut epithelial permeability	(Broderick *et al.*, 2004, 2009; Mason *et al.*, 2011)
Pea aphid: *Acyrthosiphon pisum*	Environment	Few in healthy aphids	*Staphylococcus, Acinetobacter, Pantoea*	Variable	Phloem sap	Mostly pathogenic and synthesis of signalling components that act as chemoattractant to predators.	(Harada *et al.*, 1997; Stavrinides, McCloskey and Ochman, 2009; Leroy *et al.*, 2011)

During their investigation of the association between the diet of 62 insect species and their intestinal microflora distribution, Colman *et al*. (2012) found that both the host diet and taxonomy affect the insect gut microbiome. The bacterial community in the latter study was assessed by analysing published 16S rRNA gene sequences. The investigation showed that *xylophagous* insects that decay wood exhibits the richest microbial intestinal flora, whereas bees and wasps host the least abundant bacterial types. Moreover, it was revealed that diet is the primary community diversifying factor for insect hosts that consume lignocellulose-derived substances.

Broderick *et al*. (2004) examined the effect of diet on the microbiota of gypsy moth larval midgut and revealed a variation in the microbial community according to the diet. The larvae were fed with sterilized artificial diet, aspen, larch, white oak, or willow and the midgut microbiome analysed using phenotypic and genotypic methods. It was shown that although *Enterococcus faecalis* and an uncultivated *Enterobacter* sp. were found in all larvae, irrespective of diet, the microbiota varied significantly, with the larvae fed on larch that exhibited 15 bacterial phylotypes (the highest diversity) and those fed with aspen that showed 14 phylotypes. Tang *et al*. (2012) investigated the influence of food on the gut microbiota of *Spodoptera littoralis* and *Helicoverpa armigera*, two lepidopteran pests, by feeding the larvae of *S. littoralis* with either Lima bean or barley and those of *H. armigera* with cabbage, cotton, and tomato. When the larvae were fed with Lima beans and tomato that contained cyanogenic glycosides (Lima beans) and alkaloids (tomato) (Ballhorn, 2008; Friedman, 2002), a high mortality and transient growth retardation were seen. Moreover, the microbiome composition was variable according to the diet.

Ji-Hyun *et al* (2014) examined the impact of various factors on the microbial community of gut bacteria of 305 insects belonging to 218 species in 21 taxonomic orders and using 454 pyrosequencing of 16S rRNA genes. The study revealed that the microbiota of the insects' gut is composed of Proteobacteria (62.1%) and Firmicutes (20.7%) with a higher diversity of microorganisms detected in the gut of omnivorous insects than in stenophagous insects such as carnivores and herbivores. It was suggested that the diverse profile of omnivorous insects is related to the consumption of more diversified foods that contain various bacterial species (Anderson *et al.*, 2013; Ji-Hyun *et al.*, 2014). A summary of the gut composition of 218 species in 21 taxonomic orders is shown in figure 1-2.

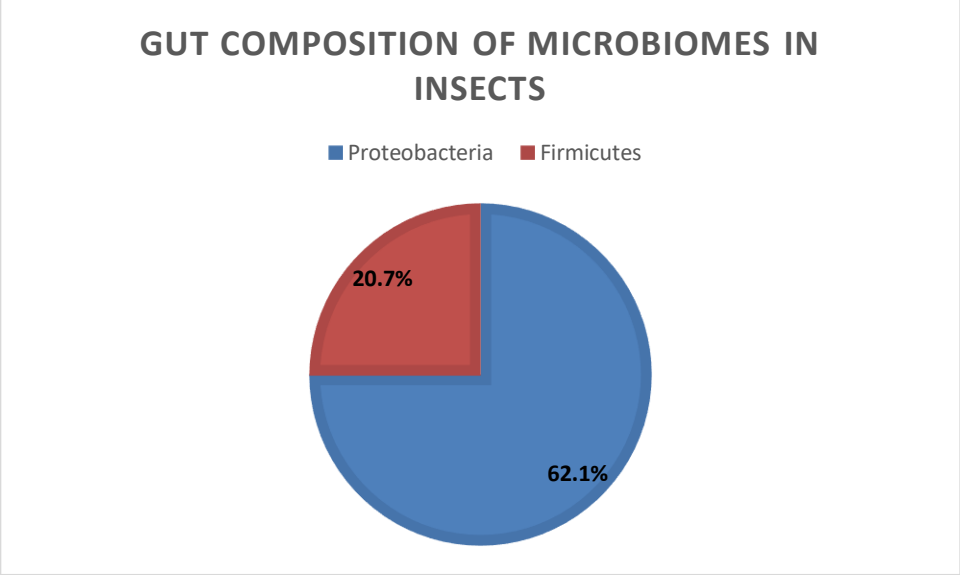


Figure 1.2: The gut composition of microbiomes in insects. Proteobacteria was the predominant microbiomes in the insect gut (Ji-Hyun et al., 2014).

It has been shown that diet supplemented with antibiotics significantly affect the microbiota of the host. An investigation by Van Der Hoeven, Betrabet and Forst. (2008) on the microbiome of *Manduca sexta* gut, revealed a diverse microbial community in insects on a normal diet and in those fed with food supplemented with two antibiotics, namely kanamycin and streptomycin. The bacterial community of insects on regular diet contained mainly Gram-positive cocci such as *Staphylococcus* and *Pediococcus* whose number significantly dropped in insects fed with antibiotics allowing the occurrence and predominance of other bacteria such as *Methylobacterium, Sphingomonas*, *Acinetobacter*, and *Paenibacillus.* The difference in the microbial community of the two types of insects was partly attributed to the susceptibility or resistance of some members of the community to the antibiotics used.

1.5 Dynamics of host-gut microbe interaction.

The caterpillar's gut is characterized by a very active epithelial transport and the highest pH value produced by a biological system (Dow, 1992). The high pH, the presence of possible antimicrobial substances secreted by the insect or derived from ingested plant tissue, the large amount of food consumed and the high competition between microorganisms suggest that bacteria capable of proliferating in this habitat could present adaptations to overcome conditions. Some of these adaptations could be based on the optimization of their enzymatic capacities, thus achieving maximum use of the various substrates available. Different studies of lepidoptera gut microbiota have shown that there is a large number of bacteria and yeast species present in their digestive tract and these microbes might have a beneficial or harmful relationship to the insect fitness (Gurung,

Wertheim and Falcao Salles, 2019). A summary of the relationship between the microbiome of the insect (beneficial or pathogenic) is shown in figure 1.3.

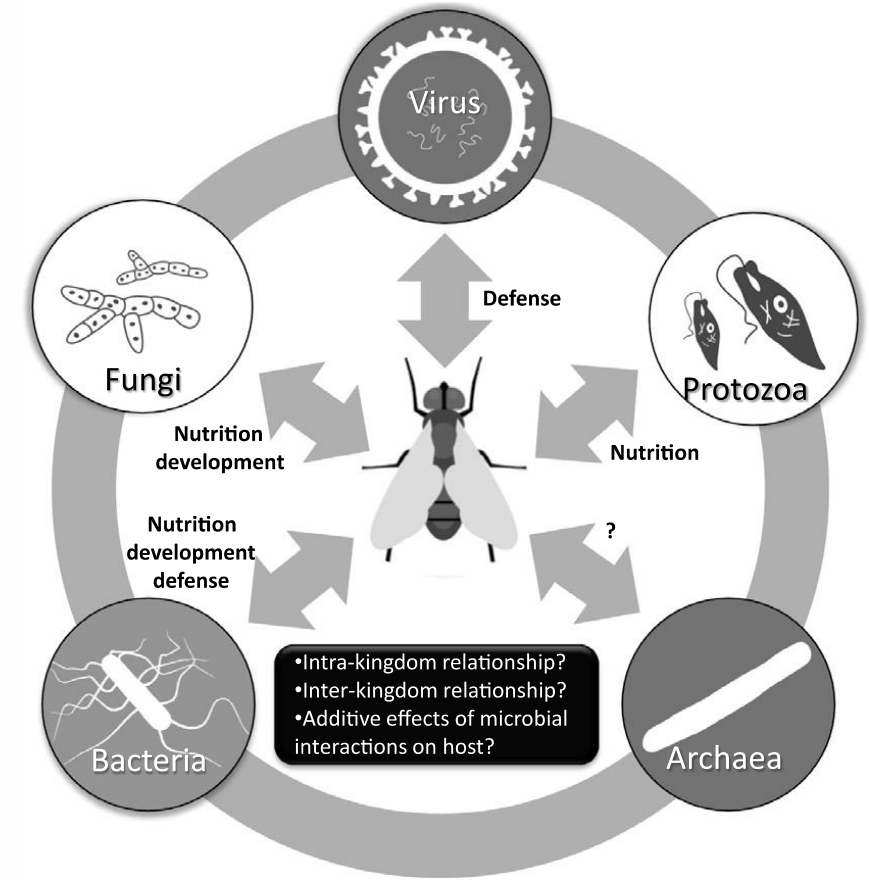


Figure 1.3: The complexity of the microbiome in insect pests. The microbiome of insect pests is comprised of various microorganisms that are connected to their host organisms (grey circle and arrows) (Gurung, Wertheim and Falcao Salles, 2019).

For instance, microorganisms present in phytophagous lepidoptera, facilitate digestion of the compounds present in plants (Vasanthakumar *et al.*, 2008). These microorganisms may have allowed the diversification of these groups of insects, in poor environments, by giving them the ability to take advantage of resources that would otherwise be inaccessible. As an example, many of the yeasts (mainly genera of *Saccharomyces*) described by these authors are involved in the digestion of e.g., xylose and hemicellulose in the intestine of these insects, which otherwise could not be used as a food resource (Suh *et al.*, 2003). The parsimonious tree showing different xylose fermenting clade (including yeast) is shown in figure 1.4.

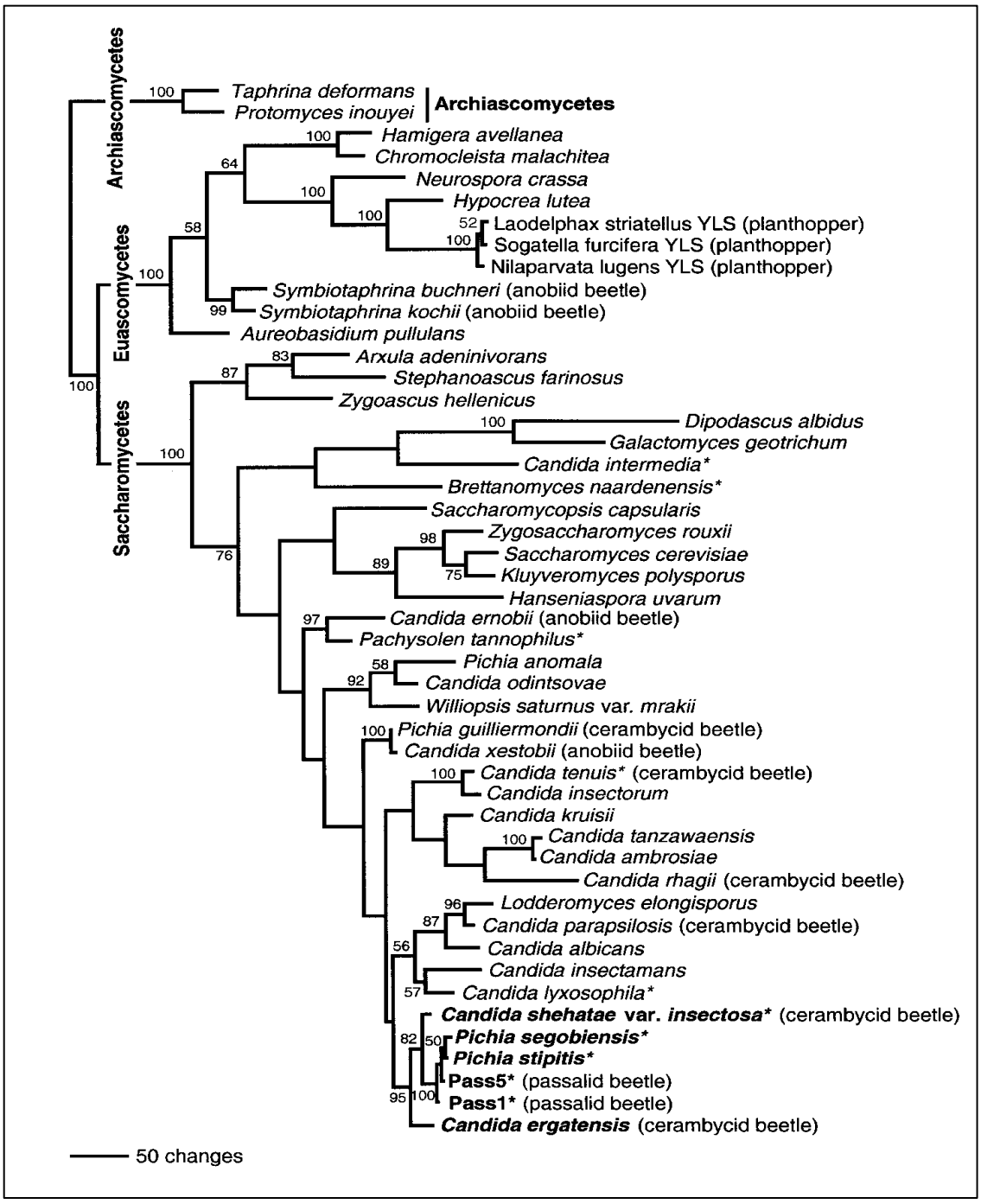


Figure 1.4: The single most parsimonious tree obtained from the combined LSU and SSU rDNA sequences. The tree only represents the xylose fermenting yeasts and other non-fermenting related groups. The xylose fermenting clade of which yeasts isolated from phytophagous (passalid beetles) are included and indicated with an asterisk (*) (Suh *et al*., 2003).

Likewise, several studies have described a bacterial population (composed of *Lactobacillus* and *Enterobacteriaceae,* among others) present in the digestive tract that could be responsible for facilitating the digestion of substrates such as cellulose and lignocellulosic compounds. Similarly, detritivorous organisms, those that feed on decomposing organic matter, require micro-communities in the digestive tract, usually composed of bacteria and fungi, which allow the absorption of certain nutrients. The degradation of the compounds that the hosts cannot digest, indicates that the microorganisms present in the digestive tract of these animals also play an important role in the digestion of organic matter.

Roukolainen *et al.* (2016) stated that many environmental factors such as diet can potentially impact the structure of the gut microbial community due to the alteration of environmental nutrients and physiochemical conditions in the gut lumen of lepidoptera. In addition, the diet and the relatively simple morphological structures of their gut contribute to the occurrence and the predominance of environmental-derived bacteria. The relative abundance of the phyla of the gut microbiome collected from field and laboratory-reared larvae is shown in figure 1.5.

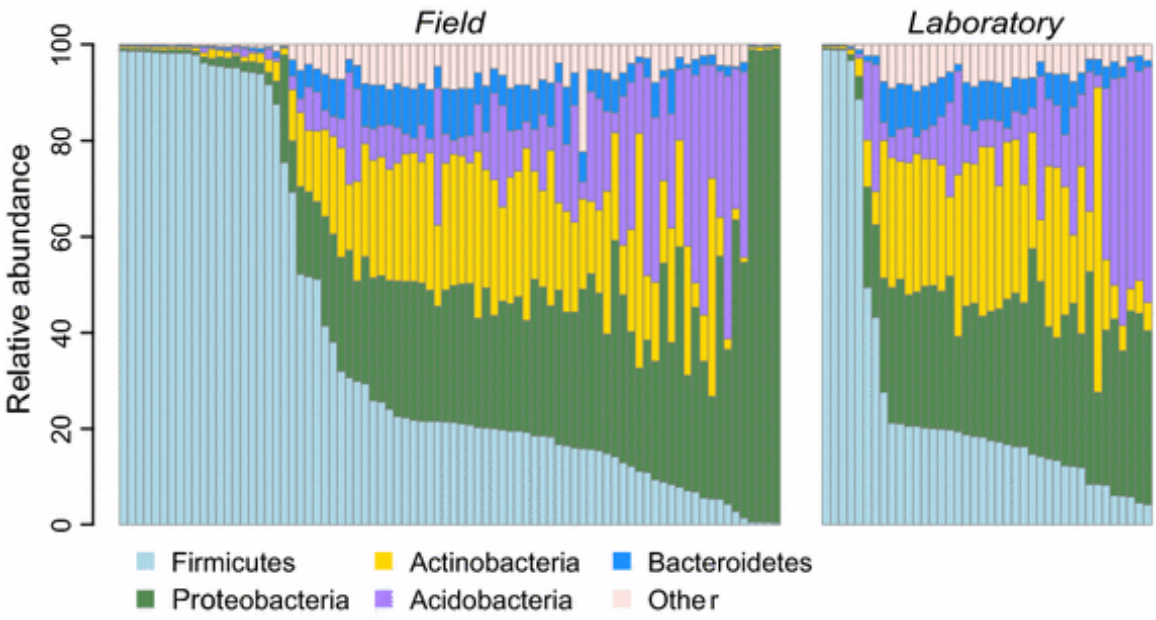


Figure 1.5: Relative abundance of the major gut microbiome phyla obtained from gut samples of field collected and laboratory reared larvae (Ruokolainen et al., 2016).

The intestinal microbiota can also have systemic effects on the growth and development of its host by modulating its hormonal signals, such as those involved in chitin synthesis that allow insects to move and grow (Goharrostami and Sendi, 2018).

In lepidoptera, the digestive tract follows a simple process, in which the food ends in the mesentery. This biological mechanism involves the function of enzymatic secretions during fermentation processes and hence active absorption of nutrients. In turn, they reach out from the mesentery to the anus whose function is aimed at extracting water, salts and minerals from the food. The microbiota in lepidoptera gives their host dietary support, even when they feed on nutrient-poor foods. These microorganisms influence metabolism and provide nutrients for the body's development and optimize the physiological and functional abilities of lepidoptera. The involvement of intestinal microorganisms in the enzymatic

cleavage of polymeric components has been demonstrated in the mesenteron and hemolymph of the larvae of certain organisms (Lemke *et al.*, 2003). It differs between a direct production of enzymes to obtain nutrients without the mediation of other organisms; and an indirect effect, resulting from mutualism with intestinal microorganisms, in some lepidoptera species (Voirol et al., 2018).

Microorganisms play an important role in the growth and development of many insect species. They contribute to the reproduction, digestion, nutrition and production of pheromones (Allen *et al.*, 2009). In some species of pesticide-susceptible lepidoptera, it has been reported that the elimination of microorganisms by antibiotics provided in a diet has reduced the susceptibility of insects to bioinsecticide (Broderick *et al.*, 2006). These results suggest that the toxicity of pesticides depends on an interaction with the microorganisms of the native intestinal community.

1.6 Methods of identification and characterization of microbial communities

Various methods are used to characterize the microbial composition of matters or living organisms. These include general and traditional phenotyping based on, e.g., morphological and biochemical characters and genotyping that are based on the analysis of genomic elements. The methods used can be culture-dependent or culture-independent. The culture-dependent techniques require the enumeration, isolation and purification of single microorganisms before their characterization and identification. The culture-independent methods do not require a prior isolation of the microorganisms. They are usually genome-based techniques where genomic elements such as DNA are directly extracted from a matrix and characterized.

1.6.1 Phenotypic methods

They are culture-dependent methods and include e.g., enumeration, isolation, purification of the microorganisms followed by their identification and characterization using conventional techniques such as Gram staining, catalase and oxidase determination, growth in different pH and salt concentration, degradation and use of various compounds such as sugars and proteins, production of toxins and resistance to antibiotics. Phenotyping is an important step in the identification and characterization of microorganisms, but it does not allow a full and reliable identification. Therefore, they are accompanied where possible by genotyping using molecular biology tools.

1.6.2 Genotypic methods

These include an array of methods that can be culture-dependent or independent. Genotypic identification of microorganisms exhibits clear advantages over phenotyping and includes fast result turn-over and improved accuracy. Methods such as polymerase chain reaction (PCR), random and amplification polymorphic PCR (RAPDPCR) allow characterization of a microbial community at the species level. Different species exhibiting the same DNA profile can be grouped and further characterized. (Liu *et al*, 1997; Daffonchio *et al.*, 1998; Da Silva *et al.*, 1999; Yamada *et al.*, 1999; Herman and Heyndrickx, 2000; Mendo *et al.*, 2000).

1.6.3 16S microbial profiling

Partial and full sequencing of various genes using specific primers have allowed the identification of the genus, species and even subspecies of microorganisms. One of the most common methods used for bacteria genotypic identification is the sequencing of the

16S rRNA gene (Clarridge, 2004). The 16S rRNA is a component of the 30S small subunit of the prokaryotic ribosome. This gene is 1500bp in length and is comprised of both highly conserved and variable regions. It contains 9 hypervariable regions that exhibit interspecific polymorphisms and permits the identification of a large number of bacteria isolates (Woo *et al.*, 2008; Yang et al., 2016). These regions termed V1-V9 have been the basis of phylogenetic classification of bacterial specie, particularly using next generation sequencing techniques. The V4, V5 and V6 regions play central roles in the translation process via binding of tRNA and interaction with the 23S rRNA subunit of the ribosome. The V2 and V8 regions play an important role in maintaining the stability of the secondary structure of the 16S rRNA gene while the role of the V3 and V7 regions in translation has not been well studied (Chakravorty *et al.*, 2007; Bukin *et al.*, 2019). The level of sequence conservation in these regions display a lot of variability with more conserved regions correlated to higher taxonomic classification and the least conserved regions utilized for the identification of bacteria at the genus and species level. The V4, V5 and V6 regions of the gene are the most conserved of the variable regions of the gene, while regions such as V3, V2, V7 and V8 of the 16S rRNA gene are the fastest evolving and less conserved (Yang et al., 2016; Johnson *et al.*, 2019). Sequencing of the full length 16S rRNA gene can reliably provide adequate information for the taxonomic classification of bacteria species. However, the short-read length of the most commonly used Illumina next generation sequencing means that often only individual V regions of the gene are sequenced and utilized for taxonomic classification purposes, for example the V4 region (Gutell, Larsen and Woese, 1994; Chen *et al.*, 2019).

Despite the usefulness of the 16S rRNA hypervariable regions for classifying and identifying various bacterial species at different taxonomic levels, there are several drawbacks of using the 16S V4 region for bacterial identification purposes. For instance, some bacterial species exhibit up to 99% sequence similarity across their entire 16S rRNA hypervariable regions, with the V4 sequences demonstrating differences at only a few nucleotides, rendering it difficult to perform an adequate classification of bacterial species at lower taxonomic levels (Poretsky *et al.*, 2014).

The approach of using 16S V4 for bacterial identification involves the generation of 16S rRNA amplicon libraries by designing barcoded sequence primers that while specific for amplification of the V4 region, contain degeneracy that enables amplification from across the bacterial kingdom, for example the universal 515F and 806R primers, see figure 1.6. Following cluster formation using the HiSeq or MiSeq platform which results in a 252bp product, quality filtering of the reads is then applied with reads shorter than 75bp being discarded. Assignment of reads to OTUs is often performed using a closed-reference OTU picking protocol, for example with the QIIME toolkit. Reads are assigned to OTUs based on their best hit to this database at greater than or equal to 97% sequence identity. Reads that did not match a reference sequence are discarded. Taxonomic assignment to each read is performed using e.g., the Greengenes taxonomy database (Caporaso *et al.*, 2012).

1.6.4 Why the 16S rRNA gene?

Reasons for utilizing the 16S rRNA gene for taxonomic classification purposes are the following: it is a highly conserved ubiquitous gene that is essential for ribosomal

translation that occurs in almost all bacteria and Archaea. Due to the highly conserved nature of this gene, universal primers can be utilized for the amplification of the 16S rRNA gene in almost all bacteria species (Janda and Abbott, 2007; Kim et al., 2014). Additionally, the ease of establishment of a complete 16S rRNA gene sequence provides essential information on the phylogenetic properties of a bacterial isolate that permits the identification of a bacteria species, often at the genus level and even at the species level. The existence of large and dedicated databases (e.g., GenBank (Altschul *et al.*, 1990)) and EZtaxon (www.ezbiocloud.net)) that contain information on almost full-length sequences of a large number of bacterial species and their taxonomic characteristics enables this identification of bacterial species. The sequences from an unknown bacteria species can be compared against the 16S sequences of already identified bacteria species that will permit the identification and the establishment of the taxonomic properties of the unidentified bacteria species. However, this approach does not always allow differentiation of closely related species due to the lack in sequence diversity of the hypervariable regions which most often results in poorly classified and species identification (Wang *et al.*, 2018). Other regions of the rRNA gene operon have been utilized for the phylogenic classification of bacterial species such as the 16S-23S rRNA internal transcribed spacer sequences (DeSantis *et al.*, 2006).

1.6.5 EZbiocloud database server

EzBioCloud, represents an up to date database based on classification of 16S rRNA gene sequences, using quality controlled 16S rRNA gene and genome sequences (Chun and Rainey, 2014; Chun, *et al.*, 2018), derived from sequencing of the genome without

contaminating DNA and the assembly of genetic information, with sequences and overall genome relatedness index (OGRI values) (i.e. how similar two genome sequences are). EZBiocloud generates descriptions of the microbiome taxonomic profile (MTP), as a basic element for the studies, which corresponds to the comparison of the sequences of the 16S gene of the sample under investigation with reference sequences established in databases. There are microbial taxonomic profiles with a large number of tera-bases in metagenomic sequences, very useful for bacterial studies today.

The identification of bacteria also progresses when the 16S rRNA gene is adjusted or combined with a database such as EzTaxon-e, which comprises sequences of bacterial strains with certified names, and is even used routinely, in addition, they add type strains of bacteria that represent species in nature. The sequences are located in the database allow the updating and reporting of species of bacteria that had not previously been identified (Kim *et al.,* 2012).

1.6.6 Primer sets used for amplification of 16S rRNA gene

There exist various primer sets designed for amplification of the various hypervariable regions of the 16S rRNA gene. According to Illumina® 16S microbial protocols, the length of the entire V4 region is ~254bp in most prokaryotes. However, the primers set commonly utilized for the amplification of V4 variable region is 515F and 806R. Figure 1.6 displays the map of primer sets used in PCR for the amplification of hypervariable regions of 16 rRNA gene (http://omegabioservices.com/index.php/16s-reference/ (Accessed 17 December 2020)).

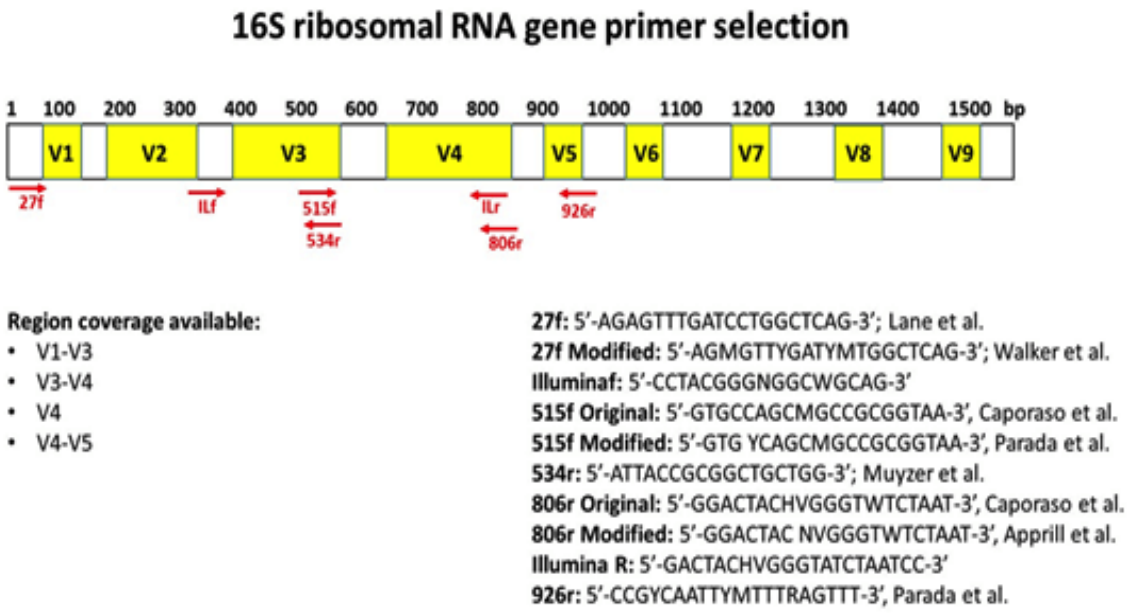


Figure 1.6: Shows hypervariable regions of the 16S ribosomal RNA gene with different and specific primer sets used in PCR (http://omegabioservices.com/index.php/16s-reference/ (Accessed 17 December 2020).

1.7 Culturomics approaches.

16S sequence identification has numerous advantages, being often definitive and it does not require the growth of each bacterium in the sample. This is important as in many cases only a small proportion of the bacteria present in a complex community will grow under standard laboratory conditions. However, it has drawbacks. Some bacteria may be important members of a community but be present at only low levels that may not be detected by 16S sequencing that is abundance dependent. Also, culture-independent methods do not produce viable cultures of bacteria that are still very valuable for studies of the biology of community members.

Culturomics represent a high-performance culture technique that establishes the multiplication of culture conditions along with rapid bacterial identification using e.g., 16 rRNA gene as a marker to describe members of bacterial populations which might not be detected by culture-free methods (Bilen *et al.,* 2018). It uses a variety of culture conditions, recognizing that standard growth assays utilize only a very narrow range of nutrients and growth conditions, are often based on short growth times and generally result in growth of only dominant sections of the total community. For example, growth times can be extended to e.g., 30 days. While fast growing bacteria are observed very quickly, slow growing bacteria can be isolated well after most others have grown and died (Browne *et al.,* 2016). Bacteria display different growth requirements; indeed, various axenic media formulations have been specifically designed for the culturing of different bacterial species that are known to thrive under such stringent conditions such as low oxygen concentrations. A diverse array of culture systems has been developed for culturing of anaerobic bacteria such as anaerobic jars, Gas-pack systems and anaerobic chambers (La Scola et al., 2014). The first step of culturomics involves the division and diversification of the samples to be identified into different culture conditions. One of the main limitations of using culturomics approach is that it might prevent the growth of the majority of bacteria present in the sample thus promoting the growth of only fastidious bacterial species. However, this is followed by the rapid identification of specific taxa using e.g., various proteomics methods such as the MALDI-TOF mass spectrometry or utilizing the sequencing of the 16S rRNA V4 region of the gene to formally identify the bacterial species present in the sample (Lagier *et al*., 2018). Culturomics has permitted the identification of a diverse array of fastidious and low abundant bacterial species that has led to an increase in the understanding of the

phenotypic classification as well as the functional role of bacterial isolates from the gut of insects (Gurung, Wertheim and Falcao Salles, 2019). For example, microbial diversity in aphids has been studied, using a wide range of sugars for carbon sources enabled the description of a certain group of bacteria, which could not be detected with a metagenomic approach (Grigorescu *et al.,* 2018). Also, Tang *et al.* (2012), evaluated the intestinal composition of *Spodoptera littoralis* and *Helicoverpa armigera*, using two culture techniques based on the sequencing of the 16S rRNA gene and microarrays, both insects were similar in abundance of bacteria, detecting *Enterococci*, *Lactobacilli, Clostridia.* Some of which are fastidious microorganisms that grow only under certain conditions.

1.7.1 Other culture-independent approaches.

Although 16S sequencing is the most widely used culture-independent technique, others are used. Denaturing gradient gel electrophoresis (DGGE) involves the separation of sequence-specific PCR-derived rRNA gene amplicons according to their mobility by applying linearly increasing denaturing conditions such as augmenting formamide/ urea concentrations (Strathdee and Free, 2013). Briefly, DNA fragments of a sample containing various microorganisms are amplified by PCR (e.g., 16S rRNA gene PCR). The resulting products are subjected to gel electrophoresis in which a constant heat (about 60ºC) and an increasing concentration of denaturing chemicals are used to denature the DNA strands. The fragments migrate and separate according to the electrical charge as well as their shape and molecular weight. At a certain point, each fragment reaches a concentration of denaturing reagents at which it melts (separation of base pairs) determined by the melting domains. Fluctuations in DNA sequences within these domains result in dissimilar melting

temperatures that force different sequences to move to different positions in the gel. DGGE has the advantage of allowing the detection of under-represented species that will not be easily recovered by culturing methods.

Non-PCR-based molecular techniques, including microarray, fluorescence in situ hybridization, and DNA-DNA hybridization are also powerful techniques.

1.7.2 Validation of and control of microbiome studies.

A major issue in conducting microbiome studies is that of contaminating DNA, particularly when investigating low-biomass samples. Even when using sterile reagents and equipment, this does not mean they are free from microbial DNA which can be carried through into the 16S PCR, generating contaminant reads in the sequence data. Validating the accuracy of studies can be done using defined mock communities as controls, for example the ZymoBIOMICS ™ mock community standard, and by including blank controls samples in which contaminating DNA can be identified and 'subtracted' from experimental samples. Eisenhofer *et al.* 2019, indicates how contaminating DNA affects the study of microbiomes. To eliminate the impacts of DNA contamination and, in turn, cross contamination in low microbial mass. Eisenhofer *et al.* (2019) proposes to minimize possible types of contamination, detect what can cause the contamination and eliminate these factors as much as possible. The author has suggested the use of 3% sodium hypochlorite, UV radiation, ethylene oxide to decontaminate reagents.

1.8 Animal models have been extensively used for studying higher vertebrate host-microbiome

The gut microbiome of mammals has emerged as a key factor that plays crucial roles in regulating the physiology and adaptation of the hosts to diverse ecological niches (Baldo *et al.*, 2019). These microorganisms also influence nutrition, detoxification of xenobiotics, activation of the gut, and the development of the host's immune system as well as the modulation of the host (Pereira and Berry, 2017). Several studies have sought to demonstrate how the gut microbiome might play an essential role in taxonomic diversity in vertebrate evolution (Sharpton, 2018), revealing that differences in gut microbiome biodiversity are correlated with the evolutionary history of vertebrates (Brooks *et al.*, 2016). Vertebrates have evolved diverse gastrointestinal features, during e.g., homeostatic colonization that allow for the natural selection of specific microbiomes collection and function, and thus lead to the establishment of host specific-species gut microbiomes signatures (Sharpton, 2018). However, the basic structure of the gut of insects is composed of three primary regions which are the foregut, the hindgut and the midgut. The primary site of digestion in insects is the midgut while the foregut might be subdivided by the diverticula for temporal food storage (Chapman, 2013). The hindgut consists of a fermentation chamber and a rectum for the storage and passage of faeces. In terms of insect gut colonization by microbial communities, the gut of the insect presents an unstable environment due to the fact that insects undergo moulting numerous times during larval growth and development which causes the shedding of the exoskeletal lining of the foregut and hindgut leading to the removal or disruption of gut microbiomes presents in these sections of the insect gut (Lehane, 1997). The midgut undergoes extensive remodelling

during moulting with the repeated shedding of the peritrophic matrix and microbial communities that are present in this tissue and in holometabolous organisms, that have distinct larval, pupal and adult stages. A complete remodelling of the gut during metamorphosis leads to an almost complete removal of the gut contents of the larvae that is ensconced into meconium in the peritrophic matrix at the pupal stage (Moll et al, 2001; Hammer, McMillan and Fierer, 2014). Microbial colonization of the gut of insects is also dependent on the physiological conditions of the lumen in the different compartments of the gut with differences in pH and oxygen availability between these compartments accounting for differences in the gut microbiome communities in insects. Studies on the midgut of the larva of lepidopterans have demonstrated that the section of the gut is extremely alkaline with pH of between 11-12 which is suitable for the proper functioning of digestive enzymes (Harrison, 2001). The pH of the gut of lepidopterans provides an environment that favours feeding and digestion of tannin-rich leaves which is an adaptative feature of the gut of lepidopterans in that it reduces the binding of dietary proteins that are ingested with tannins thus improving nutrient availability in the gut. However, this might prevent the growth of microbial communities in the gut of these insect thus demonstrating the low abundance of gut microbiomes in the gut of lepidopterans (Dow, 1992). The guts of termites have been extensively characterised and were shown to have evolved from cockroaches with distinct compartments that house different microbial communities. The hindgut of termites is composed of various compartment that contain a dense network of microbial communities that differs among the various compartments (Köhler et al., 2012). Recent innovative studies have permitted the study of the microbiome function and the evolution of the vertebrate host. This has been possible through the use of metagenomic

functional annotation studies that aid in determining and differentiating the genetic signature of the gut microbiome, which can thus lead to the identification of the different genera of microorganisms such as bacteria, fungi and other microorganisms that constitute the gut microbiome of the vertebrate host (Garud *et al.*, 2019; Fu *et al.*, 2020). Tools such as high-throughput culturing of the vertebrate gut microbiome and the transplantation of gut microbiomes into the gastrointestinal tract of vertebrate host have permitted the ability to determine the functional role of specific microbiomes on the host phenotypes (Kostic, Howitt and Garrett, 2013). Several model systems such as mice and rodents have been utilized to study the host-microbiome interactions in vertebrates, and this has permitted the manipulation of the interplay between the host and the microbiome that enables a certain degree of experimental control that will not have been possible to carry out in human subjects (Nagpal et al., 2018).

1.8.1 The fruit fly (*Drosophila melanogaster*)

The fruit fly has contributed significantly to a greater understanding of cellular functions in developmental biology studies. Considering the abundance of genetic tools that have specifically been designed to study the cellular processes in developmental biology in this model system, *Drosophila melanogaster* has been utilized to study the gut microbiome and its role in innate immunity. Numerous studies have demonstrated that the microbial communities in this model system are less complex than the gut microbiome in humans (Dionne and Schneider, 2008). An extensive study of the gut microbiome of laboratory raised and wild-type *Drosophila melanogaster* has demonstrated about 30 operational taxonomic units (OTU) found in wild-type Drosophila melanogaster. In

contrast, laboratory-reared models have, on average, only 6.3 OTUs/sample (Chandler *et al*., 2011). The most frequently identified gut microbial communities comprised the family of *Acetobacteraceae, Lactobacillales,* and *Enterobacteriaceae*, and the diet of this model system was demonstrated to be the primary determinant in the species-specific gut microbiome (Corby-Harris *et al*., 2007). The gut microbiota was observed to be obligate aerobes or aerotolerant, leading to the hypothesis that oxygen is capable of penetrating the gut of this model system and is thus required for these microbial communities to carry out their functional roles (Shin *et al*., 2011). Particularly, the potential aerobic growth and the taxonomic simplicity of the gut microbiota of *Drosophila melanogaster* has allowed the relative ease of the *in vitro* culturing of the microbial community obtained from some stocks of this model system and has permitted the insight into the relationship between the host and the symbiont (Charroux and Royet, 2012).

1.8.2 Zebra fish (*Danio rerio)*

The zebrafish is developing as a model organism for the study of the gut microbiota-host interactions (Stephens *et al.*, 2016). The 16rRNA sequencing of the gut microbiota content of wild-type and laboratory-reared zebrafish has permitted the identification of different gut microbiota classes that reside in the intestinal tract of this model system, particularly *Gamma-Proteobacteria* and *Fusobacteria*. It was thus observed that despite the differences in geographic location between wild-type and laboratory-reared zebrafish, the selection of host-microbiota present in the intestinal tract of this model system is influenced by factors such as the anatomy of the model system, the availability of nutrients, and the gut habitat effect (Roeselers *et al.*, 2011). A gnotobiotic experiment was conducted whereby the

microbiota community from zebrafish was transplanted into the intestinal tract of mice and vice versa. It was observed that the microbiota from the zebrafish underwent a transformation acquiring almost near identical characteristics in terms of structure and function of the gut microbiota of the mice. The results from these gnotobiotic experiments clearly demonstrates that the gut of the zebrafish has acquired physiological conditions that can permit the growth and maintenance of the gut microbiome colonies from the mice despite the fact that specific microbial communities persisted in the gut microbiota of zebrafish transplanted with the microbiota contents from mice (*Firmicutes* and *Proteobacteria* phyla), several gut microbiome communities were undetected (*Bacteroidetes*) in the microbiota of zebrafish transplanted with the gut contents of mice. Similar findings were also observed when mice were transplanted with the gut microbiota of zebrafish with decreases in *Proteobacteria* in the mice gut and an increase in the *Firmicute* population. *Bacteroidetes* remained mostly undetected in the gut microbiota of mice transplanted with zebrafish gut contents. This experiment demonstrates that the host microenvironment plays a vital role of physiological characteristics in host gut microbiota (Rawls *et al.*, 2006).

1.8.3 Mice (*Mus musculus)*

The laboratory mouse has been utilized as a model system for higher vertebrates to study factors related to mammalian physiology, brain development, bone mineral density, angiogenesis as well as the innate and adaptive immune system (Stappenbeck, Hooper and Gordon, 2002). They share an almost 99% gene sequence similarity with humans with key similarities in their gut microbiome being observed in the human gut microbiota as well

(Swanson *et al*., 2011). As such, this model system has been utilized to evaluate the interactions between the host and the microbiome which is applicable to the interactions in the human gastro-intestinal tract (Rakoff-Nahoum *et al*., 2004). Animal husbandry practices and the use of prophylactic antibiotics can have an effect on the composition of the gut microbiota and these effects have been utilized to study the role of the host-microbiota in studying its effect on the immune system of the host (Littman and Pamer, 2011). The effect of microbiota on the different immune cell populations and cytokines have been studied in mice. Depletion of the gut microbial community in mice has been demonstrated to cause alterations in the different cell types of the innate immune system that are located in the gastrointestinal tract and the spleen (Littman and Pamer, 2011), see section 1.9.2 regarding the use of mice model in studying the role of gut microbiome in human immune system.

1.9 *Manduca sexta* as a model for microbiome research.

Investigations of the microbiome of the gut of various insects have revealed the presence of diverse microorganisms. Insect microbiomes can be simple or complex according to e.g., the insect and the diet. One of the insects used to investigate host-microorganism interactions is *M. sexta* (Tabatabai and Forst, 1995; Martens et al., 2003; Van Der Hoeven, Betrabet and Forst, 2008), an insect pest belonging to the Lepidoptera order and the family *Sphingidae*. The name of the insect *Manduca* means glutton in Latin. It derives from the fact that the larvae have a huge appetite. The common name of this plant pest is tobacco hornworm as larvae form and the hawk moth at the full-grown adult stage. This insect feeds on plants belonging to the *Solanaceae* family, such as tobacco,

tomato, and potato. The insect undergoes a complex metamorphosis from being a small egg of about 1mm diameter. The larva which emerges after the eighth day, sheds the old exoskeleton by moulting for further larval stages, falling and digging in the soil. The pupa follows a transformation process and emerges as a full-grown moth.

M. sexta has an innate immune system involving many elements that are comparable to that of higher vertebrates (Kanost, Jiang *et al*., 2004; Eleftherianos, ffrench-Constant *et al*., 2010). Some of the immune elements include haemocytes which are capable of neutralising some microorganisms via direct phagocytosis. Furthermore, the immune response includes an activation of the phenoloxidase melanisation cascade, a humoral system that is similar to that of mammalians. *M. sexta* has 28 chromosomes and a total genome size of about 500 MB. Transcriptome sequencing of fat body, haemocytes, and midgut of the insect has been done using pyrosequencing and Sanger sequencing. RNA sequencing was also done for the immunotranscriptome analysis of the insect in various tissues namely the fat body and the haemocyte with and without immune-challenging (Gunaratna and Jiang, 2013).

1.9.1 Immune transcriptomics and the identification of the genomes sequence of *Manduca sexta*

Innate immunity plays an important role in understanding host-pathogen interactions which has been observed to have evolved in the different insect and mammalian species permitting the discovery and the classification of the evolutionary concept of animal immunity and allows for the functional comparison between diverse metazoan groups to identify unique and shared features of innate immunity (Rolff and Reynolds, 2009). The discovery of microarray, next generation sequencing technologies

coupled with bioinformatics has permitted the obtention of a large pool of immunotranscriptomics data from insects whose genomic sequence data are known such as *Drosophila melanogaster*, *Bombyx mori* and the *Anopheles mosquito* (Govind, 2008; Clayton, Dong and Dimopoulos, 2014; Jiang et al., 2019). However, *Manduca sexta* has been extensively utilized as a model to study insect physiology, detoxification, expression of innate-immune related genes in response to invading pathogens. Transcriptomic analysis of the fat body, hemocyte and midgut which displays a high expression of innate-immunity related genes was carried out in *Manduca sexta* using 454 pyrosequencing and Sanger sequencing. Based on the sequencing results, it was observed several changes in the expression of genes that were involved in the modulation of innate immunity in this species such as hemocyte adhesion, the recognition of pathogens and signal transduction/modulation. A total of 129 additional immunity-related genes were identified with genes involved in the regulation of intracellular 31% and extracellular signaling pathways accounting for 22% of the total of innate-immune genes reported in this study. The expression of genes involved in innate immunity varied in the fat body and hemocytes for this species before and after immune challenge of the larvae of *Manduca sexta*. Increases in extracellular protein transcripts was correlated to the induction of AMPs in the fat body after the immune challenge. Genes involved in pathogen recognition such as Dscam, Draper, leptin and nimrod were observed to have increased in the fat body. However, genes such as galectin-2 and TEP1/2 were downregulated in the hemocytes. Signal transduction pathways such as the Toll pathway were observed to be activated through the increased expression of the Toll receptors in the hemocytes and the fat body. Members of the Toll complex such as *MsPelle* and *MsCactus* were upregulated in the fat

body and the hemocytes. Additionally, activation of the IMD pathway was observed in the fat body through the upregulation of the IMD-FADD-Dredd complex and the downregulation of *MsTAKI* and *DmSerpernt* was observed. In terms of the activation of the MAPK-JNK-p53 pathway, *MsRac1* showed a two-fold increase in the fat body and a slight increase in *MsRas85D* in hemocytes. *MsMKK3* and *MsMEKK1* showed two-fold down-regulation in haemocytes that were not immune challenged. Components of the JNK pathway were identified in both the fat body and the hemocytes with decreased levels of these components being observed in the fat body compared to the hemocyte. In terms of haemocyte adhesion, three specific integrin subunits were identified (integrin α, β-integrin and integrin linked protein kinase). Integrin α1 was downregulated in the fat body while integrin-β subunits were mildly upregulated. Neuroglian and tetraspanin were significantly elevated in the hemocytes. Members of genes involved in autophagy were also measured in the fat body and hemocyte. It was observed an upregulation for Atg8 while other genes involved in autophagy such as Cys proteinase Atg4, Atg4 like proteins were downregulated in both the fat body and the hemocyte. Lastly, the evaluation of the gene expression of components of the AMPs pathway such as the lysozyme-like protein (LLP-1) was upregulated in the fat body with attacin as well.

This study has permitted the identification of 95 new immune-related genes, in addition to the 137 immunity-related genes have been previously reported for *Manduca sexta*, thus it brings the total number to 232 identified genes. The analysis of the transcriptome of the deep body fat and hemocytes of *Manduca sexta* demonstrated the presence of a large pool of immune-related genes that were identified by genome analysis studies. Genes involved

in signal transduction and the regulation of the innate immune system, pathogen recognition execution, cell adhesion and autophagy related (Atg) molecules were also shown to play key roles in many cellular activities along with human disease. The results from this study clearly demonstrates that genes regulating the immune pathway were mostly upregulated in the fat body compared to the hemocyte and this might be important for the construction of the immunogenome of *Manduca sexta* and needed to study and address key questions regarding higher vertebrates' disease (Gunaratna and Jiang, 2013).

1.9.2 Antimicrobial response of insects and mammals in terms of the immune system

Changes in the gene expression pattern of the gut microbiota through various experimental procedures has permitted the study of antimicrobial response of insects and mammals in terms of the immune system of the host organism. In mammals, secretory IgA and immune cell types were observed to be reduced in the intestines (Kennedy, King and Baldridge, 2018). These findings are in line with findings that changes in the gut microbiome might promote the onset of inflammatory diseases in the gut. Genes such as *NOD2* might be implicated in the emergence of Crohn's disease in humans (Balzola, Bernstein and Van Assche, 2010). Knockout of the *NOD2* gene in mice was demonstrated to cause an increase in the colonization of mouse pathogens and mice with altered expression of the *NOD1* and *NOD2* genes present with changes in the composition of the gut microbiota (Franchi, Muñoz-Planillo and Núñez, 2012). Genes such as *NLRP6* function in the inflammasome pathway, through the activation of pro-inflammatory pathways such as NF-κB which in turn regulates the expression of pro-inflammatory cytokines and

chemokines such as IL-1β and IL-18 (Elinav *et al*., 2011). Mice deficient for *NLRP6* showed alterations in the gut microbiota with microorganisms from the class *Prevotellaceae* showing great expansion and colonization of the mice intestinal tract (Brinkman *et al*., 2011). Mice were additionally observed to have increased incidence of colitis (inflammation of the gut) and this might have been due to the activation of the NF-κB pathway, which is a key activator of inflammatory processes (Brinkman *et al*., 2011). *NLRP6* deficient mice also demonstrated resistance towards certain pathogenic bacteria as well as an endogenous alteration of the gut microbiota. Caspase-1 and 3 play an important role in the activation of inflammatory processes through the cleavage of IL-1β and IL-18 which play an important role in regulating the inflammatory response in the intestinal tract of the host organism (Zaki *et al*., 2010). The results from these studies clearly demonstrated that dysregulation of the inflammatory processes might play an important role promoting changes in the microbiota of the host which might be implicated in the regulation of the inflammatory processes in the host vertebrate model.

The gut microbiome of insects shares an intimate and symbiotic relationship with the host which has led to the development of an evolutionary outcome that promotes the survival of insects under extreme environmental conditions. The acquisition of a gut microbiome by insects leads to the adaptation of these bacterial species to the gut microenvironment which have then evolved and acquired specialist functions that are essential for the survival of the host. One such important functions of the gut microbiome is the maintenance of the innate immune system, which is essential to prevent pathogenic infections that might otherwise result in the poor survival of insects (Gupta and Nair, 2020). The effect of the

microbiota in the intestinal tract of insects and the modulation of inflammatory processes has been established by carrying out studies on the host microbial interactions in insects such as the *Anopheles mosquito* as well as in *Manduca sexta* (Krams *et al*., 2017; McMillan and Adamo, 2020). The body and intestinal tract of hematophagous and non-hematophagous insects are principally colonized by microbiota of different taxa that can be either obligate or facultative symbiont (see Table 1.2). The gut of the insect does not only functions in the digestion of food but plays an important role in the innate immunity of the host. During feeding, food that enters into the intestinal tract may trigger an immune reaction from the host. However, to prevent the occurrence of an immune response of the host towards e.g., bacteria and food particles, the gut of the insects secretes AMPs in e.g., fat body and/ or midgut which might be involved in the nutritional immunity strategy (MacMillan and Adamo, 2020). It was reported in the study conducted by MacMillan and Adamo, (2020) that the gene expression levels of transferrin (iron-free protein) after 24 h oral challenge did not elevate in the midgut of *M. sexta* larvae, while it increased wherein bacteria were both directly injected to the haemocoel and orally ingested. This finding suggested that the tolerance of innate immune response of *M. sexta* towards bacteria is dependent on type and number of bacteria (MacMillan and Adamo, 2020). The immunomodulatory effect of the gut microbiome is determined via the establishment of contact between the gut microbiome and the epithelial cells lining the intestinal tract. Toll-like receptors *(TLRs*) and the nucleotide oligomerization domain (*NOD*) which are expressed on the surface of epithelial cells in the intestinal tract are capable of recognizing microbiota in the gut which leads to the activation of the innate immune response in the host intestinal tract (Hemmi et al., 2000). Lipopolysaccharides secreted by Gram negative

gut microbiota bind to *TLR-4* while peptidoglycans synthesized by Gram positive bacteria binds to *TLR-2* which leads to the activation of the innate immune system in the host cell. This clearly demonstrates that the composition of the gut microbiome might have a role to play in the regulation of the innate immunity pathway in insects and higher vertebrates (Kelsall and Leon, 2005).

Table 1.2: Symbiotic bacteria were commonly dominated within hematophagous and non-hematophagous insect species (Gupta and Nair, 2020).

Example symbionts	Association way	Insect host	Acquisition way	Position within the host	Transmission rout	Reference
Serratia entomophila	Pathogenic	Grass grubs	Feeding	Digestive tract	Toxic	Hurst et al., 2004
Klebsiella sp., *Morgnella* sp. *Enterobacter aerogenes, Bacillus cereus, Bacillus sphaericus, Serratia* sp.	Temporal association	Neuropteran	NA	NA	Toxic	Nishiwaki et al., 2008
Xenorhabdus nematophilus	Pathogenic	Wax moth	Acquired	Haemolymph	Toxic	Mahar et al., 2005
Photorhabdus luminescens	Pathogenic	Tobacco hornworm	Acquired	Haemocoel	Toxic	Münch et al., 2008
Blochmennia floridanus	Obligate	Carpenter ant	Inherited	Oocytes, midgut	Maternal	Zientz et al., 2006
Serratia marcescens	Opportunistic pathogenic	Fruit fly	Acquired	Gut, body cavity	Toxic	Nehme et al., 2007
Sodalis glossinidius	Facultative	Testes fly	Acquired & inherited	Various tissues	Milk glands, Mating	De Vooght et al., 2015
Wolbachia sp.	Facultative parasites	Different insects	Inherited	Extracellular, Bacteriocytes	Transovarial	Miller, 2013
Rickettsia sp.	Facultative parasites	Different insects	Inherited	Bacteriocytes, extracellular	Transovarial	Gottlieb et al., 2006

1.9.3 Laboratory-bred *M. sexta*

The Bath colony was established in late 1970s by Prof. Stuart Reynolds who first introduced the *M. sexta* organism as a model for different biological research interests. Importantly, the University of Bath is the only institution in the UK that is permitted by DEFRA to hold a licence for the maintenance and breeding of *M. sexta.* To date the colony is free from latent pathogens and has been well maintained by technicians with expertise and long-term experience in this field.

The caterpillar stage is a multicellular organism that has a short life span, while its size makes it easy to handle and to control under laboratory conditions. Under typical laboratory or colony conditions; 26^{o} C, 47% humidity and 16:8 light: dark period, *M. sexta* usually undergo five different larval stages from being 1mm small eggs to the last larval stages before the pupation period (Kingsolver, 2007). For the Bath colony breeding stock, the hatchling 1st instar larvae usually take 2 to 3 days before moulting to the second larval instar which is similar in the size to the 1st stage. The third and 4th stages normally take up ~5 to ~11 days respectively. Clear discrimination between the latter stages is difficult in terms of size and weight. At post 5th instar (13d) the size and weight of the larvae becomes more distinguishable which are usually estimated at the average length of ~6cm and weight ~2 g. At the late 5th stage (15d) larvae grow faster and their weight increases dramatically (~4g). At nearly the last day of the late 5th larvae stage (17d) the body size can reach up to ~ 8 cm with an average weight between 6 to 10 g before the wonderer stage (see Figure 1.7).

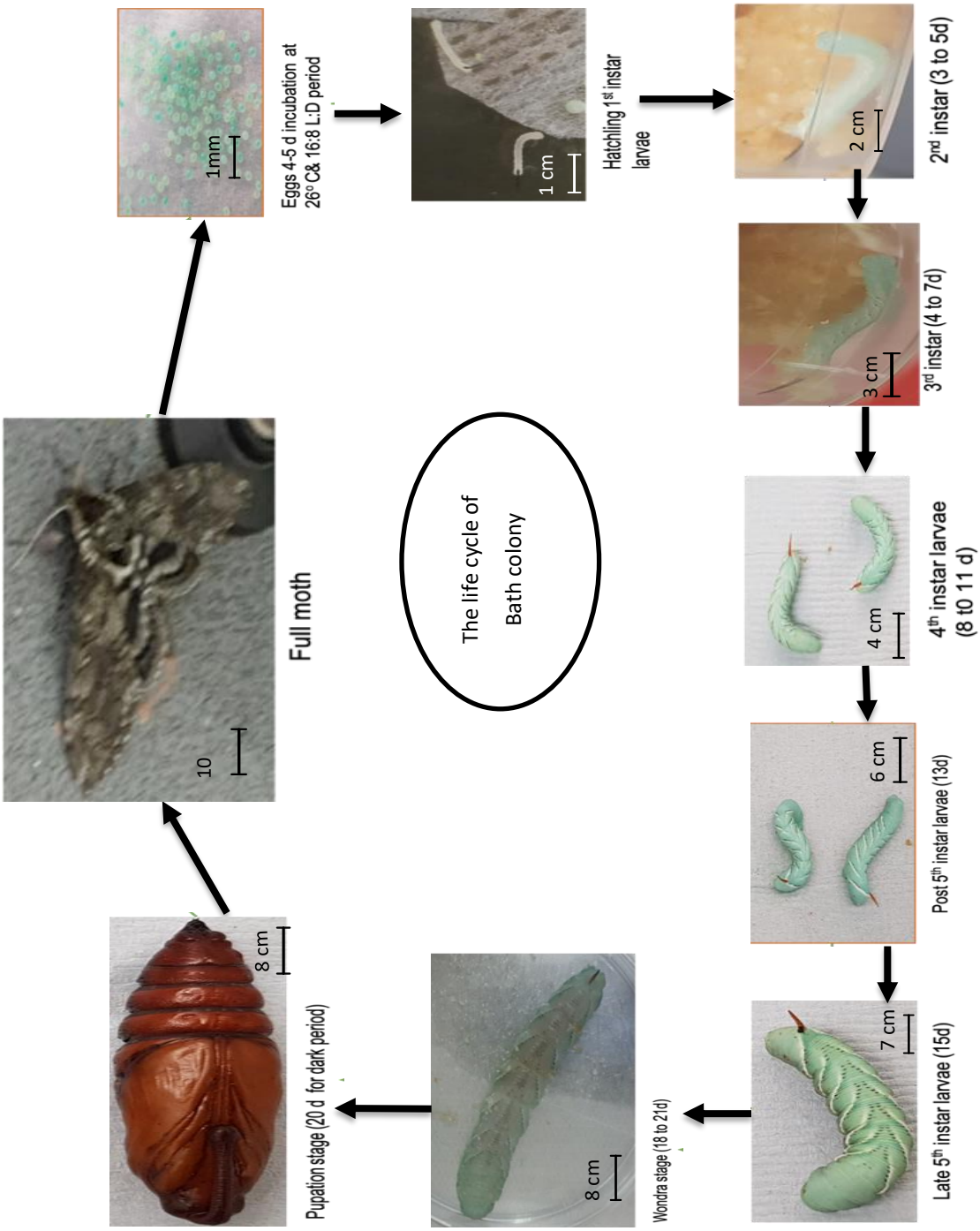


Figure 1.7: Bath colony maintains the life cycle of *M. sexta* under well controlled conditions. *M. sexta* typically undergo five different larval stages from being 1mm eggs to the full moth as shown in the above cycle. All photos presented herein this thesis are only represented the growth of Bath colony were taken during this project.

1.9.4 Studies of *M. sexta* gut microbiome

There have been several studies of the microbiome of *M. sexta*. Analysis of the gut microbiome of the larvae by PCR-single-strand conformation polymorphism (PCR-SSCP), reverse transcriptase (RT)-PCR-SSCP and stable isotope probing (SIP) revealed a diversity of gut bacteria (Brinkmann, Martens and Tebbe, 2008). In this study eggs from *M. sexta* breeding stocks were reared on whole tobacco plants. The eggs were obtained from adult insects that were maintained in a flight cage supplemented with a tobacco plant for egg deposition at 28^{o}C under a light/dark (16 h: 8 h) photoperiod. Following the hatching process and by feeding themselves with the tobacco leaves, the larvae developed and underwent all five larval stages within five weeks. Nucleic acids were extracted from whole larvae at the instar 1and 2 stages, whereas for larvae at instar 3, and 4 stages, the nucleic acids were obtained from the gut. It was possible to detect the presence of various species belonging to different genera such as *Burkholderia*, *Enterococcus*, *Citrobacter*, *Ralstonia*, *Cupriavidus*, *Enterobacter*, *Sphingomonas*, *Flavobacterium*, *Delftia* and *Bacillus*. Using the stable isotope probing it was suggested that although a diverse microflora was present, many of them were not metabolically active. To do this, larvae were reared on tobacco leaves that were grown in an atmosphere highly enriched with $^{13}CO_2$. Control larvae were reared on tobacco leaves that were grown in a standard atmosphere. Nucleic acid extracts from these larvae were used to conduct SIP and SSCP of RT-PCR products. A DNA product corresponding to *Enterococcus* sp. was produced from control samples at a decreased intensity compared to the product derived from larvae reared in ^{13}C enriched conditions. This increase in ^{13}C content indicated the presence of metabolically active bacteria that incorporated the ^{13}C during growth. The profile obtained

with the rRNA-based SSCP also supported the presence of metabolic activity based on the assumption that active bacteria contain more ribosomal particles than inactive ones. This study showed that only *Enterococcus* species isolated from the insect eggs demonstrated a clear metabolic activity in the gut as indicated. On the other hand, *Citrobacter sedlakii*, another bacterium also detected on eggs, exhibited negative rRNA-SSCP and SIP-rRNA-SSCP, suggesting that the species may not possess metabolic activity in the gut. A *Burkholderia* species exhibited metabolic properties on tobacco leaves that were used to feed the insects. However, in the gut, it failed to express its activity. Brinkmann, Martens and Tebbe. (2008) suggested that a limited diversity of metabolically active bacteria is found in the larval gut as limited species showed a positive rRNA-SSCP and SIP-rRNA-SSCP.

Bacteria that are metabolically active, dynamically use plant material and other carbon sources excreted into the gut lumen by the insects themselves and consisting of the peritrophic membrane made of chitin and proteins. The carbon and energy sources are used for various metabolic activities of the bacteria, including catabolism of polymers and production of antimicrobial peptides. The catabolism of polymers releases compounds such as simple sugars needed for the development of other bacteria, whereas, for example, *Enterococci* species known to be metabolically active are able to produce novel antimicrobial compounds against compromising bacteria present in the gut (Shao et al., 2014).

Another study conducted by Van Der Hoeven, Betrabet and Forst. (2008) investigated the effect of a diet containing antibiotics on the microbiota of *M. sexta* gut and revealed the presence of various types of microorganisms. Using a culture-dependent

approach, 16S rRNA gene sequencing and phylogenetic analysis, it was shown that the gut of larvae fed with artificial foods contained Gram-positive cocci (*Staphylococcus, Pediococcus, Micrococcus, Kocuria, Bacillus, coryneforms (Corynebacterium*)), yeast and fungi. When antibiotics (kanamycin and streptomycin) were added to the diet, a significant shift in the bacterial community was observed; the level of Gram-positive cocci dropped significantly, and the occurrence of new bacteria such as *proteobacteria*, *Methylobacterium*, *Sphingomonas Acinetobacter*, and *Paenibacillus* were observed. *Bacillus*, *Staphylococcus*, and *Microbacterium* were present in larvae fed either antibiotic-supplemented or antibiotic-free diet. Studying the susceptibility of some of the bacteria to kanamycin and streptomycin has been done by Van Der Hoeven, Betrabet and Forst. (2008) who demonstrated that the disappearance of some species in the microbiome of the gut of the antibiotic-fed larvae was related to their susceptibility to the antibiotics. The persistence and occurrence of other species in the gut of the antibiotic-fed larvae was presumptively attributed to their resistance to the antibiotics. However, some other bacteria were sensitive to the antibiotics but were still recovered in the antibiotic-fed larvae while other were resistant and disappeared. It was suggested by Van Der Hoeven, Betrabet and Forst. (2008) that the behaviour of bacteria toward the antibiotics may be in some cases different in vitro and in vivo.

In a study conducted by Mason et al. (2011), *M. sexta* was used as a model to explore the pathogenicity of *E. faecalis*. The bacterium is a normal commensal microorganism of the gut of various animals such as *M. sexta*. However, when it migrates to other organs it can cause diseases and even death in some cases. This change of status undergone by *E. faecalis* referred to as commensal-to-pathogen switch was screened. It

was shown that in *M. sexta, E. faecalis* is present in the harsh midgut of larvae without inducing any apparent adverse effects. However, the introduction of the bacteria by injection directly into the larval hemocoel was followed by a quick death. Also, a simultaneous oral administration of *E. faecalis* and *Bacillus thuringiensis* insecticidal toxin, that targets the midgut epithelium, caused a high mortality rate. Mason et al. (2011) demonstrated that the loss of gut integrity caused by *B. thuringiensis* toxin was associated with the translocation of *E. faecalis* from the gastrointestinal tract into the haemolymph. Upon arrival in the haemolymph, *E. faecalis* caused an innate immune response, shown by the appearance of haemocyte aggregation in larvae before death. The study demonstrated that *M. sexta* is an efficient model for screening the pathophysiology of sepsis induced by *E. faecalis* and the mechanism behind the death of larvae caused by the toxin of *B. thuringiensis* which is commonly used as a pesticide.

During my PhD study, it was reported that the number of bacteria in the intestines of *M. sexta* was thousands of times less than that found in other insects and vertebrates (Hammer *et al.,* 2017). In addition, there was great variability in the type of intestinal bacteria among caterpillars of the same species, which may indicate that there are no specific types or groups of bacteria in the intestines of the caterpillars (Hammer *et al.,* 2017). This suggests that those few bacteria are not residents of the intestine, they are not bacteria that multiply and live in the intestine but may be transitional bacteria which have entered with food. It was shown that the growth and development of *M. sexta* was not dependent on the activity of intestinal bacteria. The study found that the absence of microbes when using different levels of antibiotics did not affect the development and survival of the caterpillar, which continued with its normal biological cycle, without any

negative effect. Butterflies and moths (lepidoptera) represent the second largest group of insects. Other insect larvae (such as stick insects) and some worms with similar digestive systems have also been shown to lack a microbiota. The authors of this research suggest that some abnormalities of the intestines of the caterpillars and other insects make this an unfavorable environment for bacterial growth and colonization.

1.9.5 Germ-free animal models

To know the basis of mutualism in a symbiotic relationship, it is necessary to carry out studies that can elucidate the mechanism and operation of each of the individual elements in this interaction. Germ-free animals' models have been important for such studies by identifying differences in the host in the absence of microbiota. For example, bacteria were eliminated from ants, to investigate the role of the bacteria in the production of compounds important for the development and the maintenance of the host physiology (Engels and Moran, 2013).

Using germ-free models, is made it possible to determine the effect of microbiota on, e.g., intestinal transit time, immune response, food intake, vitamins, susceptibility to infections and even behavior has been studied. The insect gut microbiome also plays a fundamental role in the synthesis of sterols, since the species of the insect groups, unlike most other animals, cannot synthesize the precursors of these compounds and for this they need symbiotic microorganisms.

Insects and plants have coexisted for more than 400 million years. In this sense, there is a microbiota associated with both organisms that has also evolved in parallel and has been able to modify the plant-insect relationship (Sugio *et al.*, 2014). Each insect has a certain

composition of bacteria with specific functions, for example, in the production of energy from the degradation of dietary fibers or the formation of certain vitamins (Douglas, 2015). The strategy is not only to characterize hundreds of species of bacteria, but to take advantage of DNA analysis and comparisons with databases to identify genes that reveal the presence of different microorganisms. The fact that there are bacterial genes associated with different characteristics according to the phases and physiological moments of the organism (Pell *et al.*, 2006).

1.10 Aim of this thesis.

Currently, there is enormous interest in the role of microbiomes of animals in health and disease. Unfortunately, to investigate the role of a microbiome in host health, a model comprising a microbial flora and host is required. Some studies can study the interaction between particular microbes and particular host cells in vitro but microbiome research is focused on the role of microbial communities in whole systems and as such animal models, particularly mice, are at the forefront of microbiome research. While these models are powerful as they are direct tests of the interplay between microbial communities and host systems, one of their drawbacks is the enormous complexity of the microbial populations, for example the mouse gut microbiome contains thousands of different species of bacteria, and of the mouse host. A simpler model would enable key tenants of the microbe-host interplay to be investigated and evaluated.

The larval stage of *Manduca sexta* is an established model organism for the study of regulation of development, neurobiology and some studies of microbial pathogenesis. It is relatively simple to breed under laboratory conditions, has a fast life cycle of around 30

days and its large size (up to 80mm) makes it easy to handle and manipulate. However, because of its potential as an agricultural pest, its breeding and use is regulated in the UK by DEFRA. The Department of Biology and Biochemistry at the University of Bath has the only license in the UK to maintain *Manduca sexta* and has established expertise in breeding and maintaining these organisms for teaching and research and for supplying other labs in the UK and Europe with material for their own studies. While several studies have investigated the microbiome of *M. sexta*, a clear role for its gut microbiome in host health and development has not been defined (Voirol *et al.*, 2018). This project is aimed at investigating the use of *M. sexta* as a model for microbiome research.

Specifically, it aims to:

1. Characterise the gut microbiome of the Bath colony of *M. sexta* larvae, and its stability.
2. Investigate the role of the gut microbiome in the growth and development of the larvae.
3. Investigate the effect of manipulating the gut microbiome on *M. sexta*, including immune function.

2.Chapter 2 MATERIALS AND METHODS

2.1 Preparation of *M. sexta* standard colony food.

The food used to feed larvae of *M. sexta* in the Bath colony contained the following ingredients; agar (22.5g), wheat-germ (2700g), casein (1260 g), sucrose (1080 g), dried active yeast (540 g), Wesson's salt (360 g), cholesterol (72 g), ascorbic acid (54 g), Vanderzant vitamin (0.2g), choline chloride (36g), 4ml linseed oil, 4ml corn oil and methylparaben (54g) dissolved in 1.650 L of distilled water and supplemented with chlortetracycline hydrochloride (0.2g) and 8ml of 1:10 (V/V) formaldehyde. Antibiotic-free food was made by omitting the supplementation with tetracycline.

The food was prepared as follows: the water was first boiled; yeast product was deactivated by heating in the microwave at low power for about 5 minutes (with stirring every two minutes). The agar was dissolved in a small amount of water in the microwave. The dry ingredients were then mixed with dissolved agar and the boiled water in a clean electrical mixer for 10 minutes. Next, the product was cooled to 50°C. The Wesson's salt, vitamins, oils and methylparaben were mixed and added. The food mixture was poured into foil trays and allowed to cool to room temperature, wrapped tightly, transferred into dated plastic bags and stored at 4°C until used.

2.1.1 Sterile antibiotic-free food (SA-free food)

Wheat germ, agar, casein, cholesterol, and water were autoclaved at 121 ºC for 15 minutes and cooled down to 55ºC in a water bath for 1 hour. Next, all other essential ingredients

were weighed out, dissolved in a small amount of sterile distilled water and then filtered through a 0.22µm syringe filter prior to the supplementation of the food (see Table 2.1).

2.1.2 Sterile antibiotics cocktail supplemented food (SA-Food)

Food was made as above but it was mixed thoroughly with a filter-sterilised cocktail of ampicillin 100µg/ml, erythromycin 1µg/ml, tetracycline 12.5µg/ml and daptomycin 1µg/ml see Table 2.1 and 2.2). Food aliquoted into sterile 50 ml Falcon tubes and kept in the fridge at 4º C for use.

Table 2.1 The composition of sterile food.

Ingredient	Weight (g)	Sterilization method
Wheat-germ	44.8	Dissolved in H_2O, autoclaved 121°C for 15 m
Agar	4.5	Dissolved in H_2O, autoclaved 121°C for 15 m
Casein	17.24	Dissolved in H_2O, autoclaved 121°C for 15 m
Dried yeast	7.3	Deactivated in the microwave for 5 m, dissolved in sterilized distilled water and filtered (0.22µm)
Wesson salt	4.9	Dissolved in sterilized distilled water and filtered (0.22µm)
Sucrose	14.77	Dissolved in sterilized distilled water and filtered (0.22µm)
Methyl 4-hydroxy benzoate	0.425	Dissolved in sterilized distilled water and filtered (0.22µm)
Choline Chloride	0.493	Dissolved in sterilized distilled water and filtered (0.22µm)
Cholesterol	0.985	Autoclave 121°C for 15 m
Sorbic acid	0.737	Dissolved in sterilized distilled water and filtered (0.22µm)
Ascorbic acid/Vanderssant	0.2	Dissolved in sterilized distilled water and filtered (0.22µm)
Corn and Linseed oils	0.5 ml each	Filtered (0.22µm)
Autoclaved distilled water	Up to 400 ml	Autoclave 121°C for 15 m

Table 2.2. Antibiotics used to produce antibiotic cocktail-supplemented food.

Antibiotic	Major target of bacterial cell	Stock mg/ml 1000X
Ampicillin	Cell wall synthesis inhibitor	100
Tetracycline	Protein synthesis 30S inhibitor	12.5
Erythromycin	Protein synthesis 50S inhibitor	1
Daptomycin	Disrupts cytoplasmic membrane	2

2.2 Conventional rearing of *M. sexta* in the Bath colony.

2.2.1 Conventional decontamination of Bath colony *M sexta* eggs.

M. sexta eggs were routinely decontaminated using the following treatment: the fresh eggs are collected from the adult winged-moth-cage every morning in regular clean pots fitted with a breathable lid. The pot with an open lid is then placed inside a large sandwich box and a smaller pot containing ~10 ml of 5% formaldehyde is placed inside. The sandwich box lid is then sealed and left for 4 hours. Next, the small pot is replaced with a new pot containing ~ 10 ml of 0.625 % ammonia solution, and the lid of the box is replaced with another one, sealed and left for 15 minutes. The eggs are taken out of the box and left inside the laminar air follow for 5 minutes to dry. The pot containing clean eggs is closed by its breathable lid and incubated at 26^{o} C under 16:8 hrs light: dark periods for 5 days until the hatching stage.

2.2.2 Rearing conventional larvae.

The hatchling 1st instar larvae are individually placed on ~ 20g of the standard food inside small conventional, clean and dated pots with breathable lids until they become post 5th instar (13d). The growing 5th stage larvae are then transferred and placed onto ~ 40g of the

standard food in larger, clean and dated pots fitted with breathable lids. Subsequently, larvae incubated under above colony conditions for further larval stages.

2.3 Sterilisation of *M. sexta* eggs.

A batch of fresh *M Sexta* eggs was collected early in the morning from the adult winged-moth cage at the Bath colony in a clean disposable plastic container fitted with a breathable and closed lid. All work regarding bacteria-free *M. sexta* was conducted inside laminar air follow safety hood class II. Approximately 100 eggs on a tissue were aseptically cut and placed inside the sterile and disposable top unit of a 0.45 μm filter vacuum unit, using sterile and disposable plastic forceps and scissors. Eggs were then exposed to 250 ml of 0.6% sodium hypochlorite solution for 3 minutes with occasional stirring. Next, the vacuum was turned on to drain the bleach solution. Immediately, eggs were washed three times with 250 mls of autoclaved distilled water.

Eggs were aseptically transferred into a sterile petri dish using sterile disposable plastic forceps. The sterile eggs were left for 30 minutes inside the sterile cabinet to dry and then aseptically transferred onto sterile BHI agar medium that was subsequently incubated under the typical *M. sexta* colony conditions at 26^{o}C and light/dark period (16h: 8h) until hatching stage for ~4 to 5 days. Eggs from the same batch treated in a similar fashion but with only autoclaved distilled water were used as a control for hatching frequency of the eggs.

2.3.1 Rearing bacteria-free *M. sexta.*

Sterile food was placed in sterile 50ml Falcon tubes fitted with 0.22µm filters in the lids to allow gaseous exchange (See Figure 2-1). Sterilised eggs were placed on the food and the tubes were incubated at 26^{o}C and light: dark 16h:8h period.

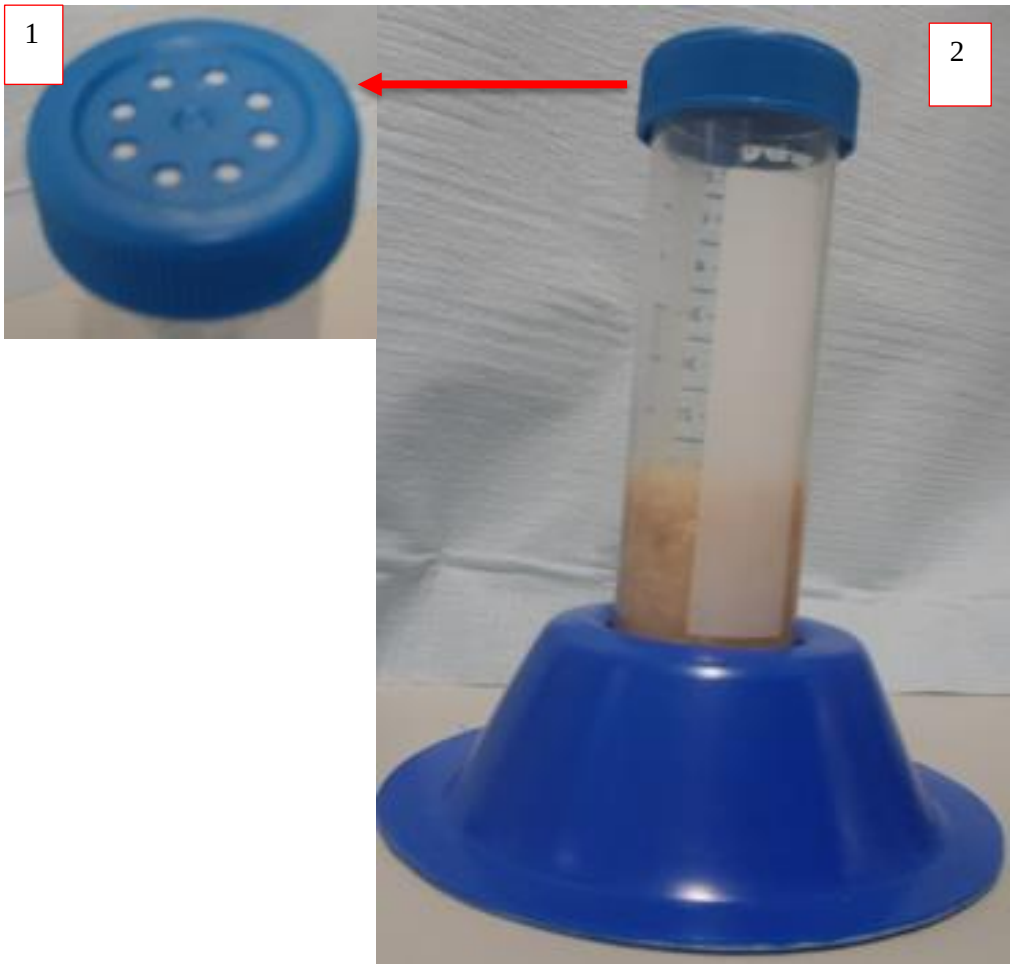


Figure 2.1: The image shows features of sterile/disposable 50 ml falcon tubes used to rear bacteria-free larvae. 1. The lid was fitted with a 0.22µm filter to allow gas exchange. 2. The tube containing 20 mg of sterile food.

2.3.2 Sterility assessment of bacteria-free experiment

To test the sterility of samples including the food, eggs as well as hatchlings bacteria-free 1st instar larvae, however, the following were carried out:

For bacteria-free eggs and the following hatchlings 1st instar larvae, however, up to 5 whole eggs or larvae were randomly and individually inoculated into sterile enrichment BHI broth (for aerobes), and TS broth (for anaerobes) using sterile/disposable forceps. Subsequently, the OD at 600nm of the inoculated broth cultures was measured and recorded every 24h in which 100µl was plated onto BHI agar (aerobes) and TS agar media (see Table 2-5).

Similarly, samples (3-5 gm) of the sterile food were randomly collected from food patch using sterile disposable plastic loops, inoculated into enrichment broth media and further incubated as described above.

2.4 Collection and treatment of larval gut contents

For collecting gut contents of larvae, the caterpillars were placed on ice for 15 minutes, and the weight of the caterpillar was recorded. Each caterpillar was disinfected by cleaning their surface with 70% ethanol followed by washing with sterilized distilled water. The gut fluids were retrieved inside sterile Petri plates by dissecting the caterpillars with sterile scissors and collecting the fluid into sterile10ml tubes.

2.5 Growth of gut bacteria.

2.5.1 Media and growth conditions.

The media used were prepared as instructed by the manufacturer and autoclaved before use (Oxoid, Basingstoke, UK.). The agars and incubation conditions used to grow gut bacteria are shown in Table 2.3 in the next page.

Table 2.3. Agars and growth conditions for the recovery of gut bacteria.

Media	Temperature	Atmosphere	Time	Microorganism	Other
Nutrient agar (NA)	30 °C	Aerobic	24-48h	General Gram+ and Gram- bacteria	----------
Blood agar (BA)	30 °C	Aerobic	24h	General Gram+ and Gram- bacteria	Hemolysis
Brain Heart Infusion agar (BHI)	30 °C	Aerobic	24h	General Gram+ and Gram bacteria	A rich medium for growth of fastidious bacteria.
de Man, Rogosa & Sharpe agar (MRS)	30 °C	Aerobic	48-72h	Lactic acid bacteria and another anaerobe	Can be incubated under anaerobic atmosphere to suppress the growth of particular Gram-positive bacteria.
MacConkey agar (MA)	30 °C	Aerobic	48h	Mainly Gram- bacteria	pH indicator, colour changes from pink to yellow (lactose fermentation)
Nutrient agar	30 °C	Anaerobic	72h	Various anaerobic bacteria	----------
Blood agar	30 °C	Anaerobic	48-72h	Various anaerobic bacteria	----------

2.5.2 Inoculation and incubation

100 µl of gut content samples were plated. All plates were incubated in aerobic conditions at 30 °C for 24-48h. Additionally, NA and BA plates were incubated at 30 °C for 24-48h

in anaerobic conditions in a 2.5 L anaerobic chamber containing one anaerobic sachet (Oxoid®) as shown in table 2.3. Duplicate plates were inoculated for each medium.

2.5.3 Enumeration

After the incubation time (24-72 h), the flora on each type of agar was enumerated. The colonies were counted, and the number of cells calculated using the number of colonies, the dilution and the volume of inoculation. The number of bacteria was expressed as CFU/ml otherwise it referred to as a number of colony (if it was inconsistent with respect to its group/dilution).

2.5.4 Isolation and purification of bacteria

Bacteria were isolated from all media where the colonies were separated. The characteristics (e.g., size, colour, the regularity of the edge) of the colonies were recorded, and 2-3 isolates of each different type of colony observed were isolated and streaked on the corresponding agar. The plates were incubated as above. Where pure culture was obtained, the isolate was stored in 20% glycerol at -80°C until required for further analyses.

2.6 Identification of the bacteria by 16S RNA gene sequencing

2.6.1 DNA extraction protocol

A single colony of each isolate was transferred separately to its corresponding agar medium and incubated at 30°C for 24 hours. Up to 3 or 5 individual colonies were transferred into a sterile nuclease-free 1.5ml and the DNA extracted according to the procedure recommended in the High Pure PCR Template Preparation Kit (ROCHE). In

the last step of the procedure, the genomic DNA was eluted in 200 µl of elution buffer and stored at -20°C until required for further analysis.

2.6.2 Polymerase Chain Reaction (PCR) used for Sanger 16S rRNA gene sequencing

The 16S gene of each bacteria was amplified from genomic DNA by PCR using the universal primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'TACCTTGTTACGACTT-3') (Weishurg et al., 1993). The PCR mixture (50 µl) contained 25 µl of 1x One 2X Taq Standard Reaction Buffer (New England Biolabs), 10 mM dNTP, 22µl of nuclease water-free, 1µl of the (20 pmol) forward primer, 1µl (20pmol) of the reverse primer and 1µl of genomic DNA. A positive control isolate (PXT E. coli) was also screened to monitor the success of the DNA extraction and amplification reaction. The PCR was performed in a thermocycler according to the conditions described in Table 2.4.

Table 2.4. PCR amplification conditions.

Steps		**Temperature** (℃)	**Time (minutes)**
Initial denaturation		94.0	0.30
35 cycles	Denaturation	94.0	0.30
	Annealing	48.0	00:30
	Extension	68.0	00:30
Final Extension		68.0	05.00
Hold		4.0	-

2.6.3 Gel electrophoresis

Gel electrophoresis was performed to determine whether PCR products had been amplified. The gel (0.8%, W/V) was prepared by dissolving 0.8g of agarose powder (Oxoid) into 100 mls of 1% Tri-acetic acid- EDTA (TEA) buffer. 4µl of ethidium bromide (Biorad®) was added per 100mls of agarose to allow the visualization of the PCR products under UV light. 5µl of PCR reaction was mixed with 1µl of (6X) Gel Loading Dye (New England Biolabs®) and loaded into the gel. A DNA marker (7µl of 1 Kb ladder) was added to the first well of the gel as a standard. The gel was run at 100 Volts for 30 minutes and the DNA profile observed under a UV transilluminator.

2.6.4 Purification of the PCR products

PCR products (1500bp) were purified using the E.Z.A.® Cycle Pure Kit (Omega Bio-Tek Inc.) according to the manufacturer's guidance. PCR products were eluted in 50µl of elution buffer and frozen at -20°C until required for sequencing.

2.6.5 Sequencing procedure

The sequencing of all purified PCR products was conducted by Eurofins Medigenomix (GmbH, Ebersberg, Germany) using the Mix2Seq Kit. The reaction mixture (17µl) contained less than 10 ng/µl DNA template and 20 pmol/µl of the forward primer. The sequences were reported in FASTA format.

2.6.6 Sequence analysis

The bacteria were initially identified by analyzing the sequences against the GenBank database using the Basic Local Alignment Search Tool (BLASTn) (Altschul et al., 1990)

of the Nation Centre for Biotechnology Information (NCBI). The identification of the isolates was based on the similarities between the query sequence and the top hit from the BLAST search. However, since GenBank contains sequences of both type strains and non-type strains that may not be correctly identified, the identification may lead to misidentification. Thus, the EZ-taxon server, another tool, which contains a manually curated database of type strains of prokaryotes and provides identification tools using a similarity-based search (www.ezbiocloud.net) was used to generate more definitive identification.

2.7 Treatment larval gut content samples.

2.7.1 Bespoke pestle device.

A bespoke pestle device for use in 50ml Falcon tubes was created in the workshop of the Engineering department, University of Bath, that can be autoclaved and was re-useable with multiple samples. The pestle is a stainless-steel unit that connected to an electric drill and fits into sterile disposable 50ml falcon tubes utilized as a mortar. The homogenised gut content samples were then serially diluted and plated as described above.

2.7.2 Use of 1mm glass beads to homogenise the larval gut content

The retrieved gut content was added into sterile, disposable and labelled 10 ml Falcon tubes containing a small amount of 1mm autoclaved glass beads. The tube was then vortexed at maximum speed for 3 to 5 min. The homogenised gut composition specimen was serially diluted, plated and incubated as mentioned before.

2.7.3 Use of a marker bacterium

E. coli XL1-Blue was stored at -80^{o} C in PBS/20% glycerol. It was streaked onto LB agar and incubated at 30^{o} C for 24 h. Several colonies (3 to 5) were inoculated into sterile 1.5 ml microtube containing 1ml of PBS buffer to obtain bacterial suspension whose OD_{600nm} was measured and recorded. The suspensions were serially diluted and 100µl of each dilution plated on LB agar and incubated at 30^{o} C for 24 hours. The colonies on plates containing a countable number of individual colonies were counted and the CFU/ml of the original suspensions was calculated. The CFU/ml of three independent suspensions was averaged and used to determine the standard CFU/ml of a suspension of OD_{600}=1.0 of the marker bacterium.

Plate grown *E. coli* XL1-Blue were resuspended in PBS and the OD_{600} adjusted to 1.0. 100µl of the suspension was added to larval gut content samples. These were serially diluted and plated onto LB agar plates and incubated at 30^{o} C for 24h. The recovered bacterial colonies were enumerated from plates that contained a countable number of well separated colonies. These values were used to calculate the percentage of the added bacteria that were recovered.

2.8 Isolation of bacterial cells from gut content samples.

Differential centrifugation was used to attempt to isolate bacterial cells away from gut content matter. 5 volumes of PBS were added to gut content samples in sterile 50ml Falcon tubes and vortexed for 2-3 minutes. The resulting suspension was centrifuged at 1000xg for 1 minute at room temperature to dissociate microbial cells from relatively large particles

of the gut content. The supernatant was collected into a new sterile 50 ml Falcon tube. The OD_{600} of the supernatant was measured and recorded to enable normalisation of the volume used to inoculate enrichment broth cultures (see below). The remaining supernatant was transferred into a sterile (29 X 104mm), 50ml Round bottom Oak Ridge style tube and centrifuged at 4^{o} C, 13 000 xg, for 5 minutes. Pellets were resuspended in 5ml PBS and centrifuged again. These steps were repeated until the supernatants became clear. The resulting microbial cell pellets were used for gDNA extraction.

2.9 Enrichment broth culture-dependent method

Two types of commercially available broth media were used during this research to culture bacteria from the gut content samples. These were Brian Heart Infusion (BHI), and Tryptone soy broth media (Oxoid). However, according to the manufacturer, the BHI broth medium includes various nutrients that promote the growth of fastidious but mainly aerobic bacteria. For the isolation of anaerobes, TS broth medium was overlaid with ~1ml of sterile mineral oil. *Clostridium sporogeneses* (ATCC 19404) is an obligate anaerobe which was used to validate that anaerobic conditions were achieved using this method. Cultures used 10 mls of broth in 15 ml sterile labelled falcon tubes. The supernatant from gut content samples following the initial low speed spin (see above) was used to inoculate broths. Cultures were incubated at 30^{o}C, for different times see Table 2.5.

Table 2.5. Incubation times for different enrichment broth cultures.

Broth medium	Condition	Incubation time (days)	Temperature
TS (anaerobic condition)	Anaerobic (static, inside conventional incubator)	3 to 5 d	30º C
BHI (aerobic condition)	Aerobic (shaker incubator)	1 to 3 d	

To inoculate enrichment broths, the volume of gut content suspension supernatant, following the low-speed spin (see above) required to inoculate 10mls of broth at and OD600 of 1.0 was centrifuged at 13,000xg for 1 minute. The resulting pellet was then resuspended in 1 ml of the appropriate broth and made up to 10mls with broth. The initial OD_{600} of the inoculated 10 ml broth was measured and recorded. Next all broth culture tubes were incubated at 30˚ C under their corresponding condition (see Table 2.5). The OD_{600} of the cultures were recorded every 24h.

2.10 Genomic DNA extraction from enrichment broths and gut content samples.

2.10.1 Testing the efficiency of different gDNA extraction kits.

Three different commercially available kits developed for extraction of gDNA from microbiome samples were tested (see Table 2.6). These kits included a bead-beating step recommended for increasing the yield of gDNA extracts from different microbial cells in a wide variety of samples. The kits were tested on an artificial mock microbial community (ZymoBIOMICS ™ mock microbial community standard, cat# D6300) comprising a defined population of different bacteria, that is widely used as a standard in microbiome experiments. A vial of the mock community was thawed on ice and vortexed for 2 min. Three individual samples of 0.75 ml each of the stock were separately transferred into three 2 ml tubes containing beads from each kit. Each sample was mixed with the corresponding lysis buffer from each kit, vortexed for 15 minutes using vortex GENIE® 2 device (Cat# 444-0486P) supplemented with a horizontal holder-adaptor disc which holds up to 24 sample microtubes (Cat #444-1045). The tubes were centrifuged at 15 000 xg for 2 minutes. The supernatants were taken into the relevant gDNA extraction protocol for each kit.

At the final step, the pure gDNA was eluted in 50ul nuclease-free water unless otherwise indicated by the manufacturer. The gDNA was stored at -20^{o} C for later use.

For extraction of gDNA from bacterial cell pellets isolated from gut content samples (see figure 4.4 chapter 4), the pellets were resuspended in 1ml of PBS in a microcentrifuge tube and centrifuged at 13000 xg for 1m. The resulting pellet was resuspended in 250 µl of DNA extraction kit buffer (QIAGEN DNeasy soil power kit) and transferred into a new labelled

tube containing extraction kit beads and gDNA extracted according to the manufacturer's instructions**.** The pure gDNA was eluted in 30 ul of elution buffer and kept at -20^{o}C for later use.

For extraction of gDNA from enrichment broth cultures, the 15 ml Falcon tube containing enrichment broth culture was vortexed for 1 min. 1ml of each culture was transferred into a sterile 1.5 Eppendorf tube and centrifuged at 13000xg for 1 min. The supernatant was discarded, the pellet resuspended in 1 ml PBS and centrifuged at 13000xg for 1m. The pellet was resuspended in 0.25 ml of kit lysis buffer and gDNA extracted.

Blank controls. To account for contaminating DNA that is present in most reagents blank controls were processed in each experiment. Volumes of PBS or sterile broth media were processed for gDNA extraction in the same manner as other samples.

Table 2.6. Different gDNA extraction kits were tested for extraction of gDNA from ZymoBiomics mock microbial community.

Kit tested	Catalogue number	The recommended amount of sample	Recommended samples
QIAGEN DNeasy soil power kit	12888-100	0.25ml	Soil, blood and gut fluid
ZymoBIOMICS™ DNA Miniprep Kit	D4300	0.2ml	Stool, soil, urine and body fluid
PureLink™ Microbiome DNA Purification Kit	A29790 Pub. No. MAN0014334 Rev. A.	0.2ml	Body fluid such as blood, soil, and body fluid

2.10.2 PCR of the amplification of V4 region of the 16S rRNA gene

Table 2.7. PCR reaction tube.

Reagent	Volume (µl)	Final concentration
2X Master Mix PCR	25	1X*
10µM forward primer	1	0.2µM
10 µM reverse primer	1	0.2µM
DNA template	Variable	< 500 ng/ run
Nuclease free water	Up to 50	

According to the manufacturer, 2X master mix gives a final concentration of 1.5 mM $MgCl_2$ and 0.2mM of each dNTP.

A set of barcoded universal 16S V4 primers, 515F–806R (Caporaso et al., 2012), was obtained from Sigma Aldrich. The PCR conditions used to amplify the 16S V4 region is described in Table 2.8. The source of primer sets: https://media.nature.com/original/nature-assets/ismej/journal/v6/n8/extref/ismej20128x2.txt

Sequence field description (space delimited):

1, Reverse complement of 3' Illumina adapter

2, Golay barcode

3, Reverse primer pad

4, Reverse primer linker

5, Reverse primer

CAAGCAGAAGACGGCATACGAGAT XXXXXXXXXXXX AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT

The PCR reaction mixture and library preparation of the 16S amplicon were conducted in the Milner centre at the University of Bath.

Table 2.8. PCR conditions for amplification of the V4 region of the 16S rRNA gene.

Steps		**Temperature** (°C)	**Time (minutes)**
Initial denaturation		95.0	3
30 cycles	Denaturation	94.0	0.30s
	Annealing	55.0	00:30
	Extension	72.0	00:30
Final Extension		72.0	05.00
Hold		4.0	-

2.10.3 The analysis of the 16S v4 rRNA gene sequence data generated from Illumina Miseq platform.

The extracted gDNA of 5th instar larval mid-gut content samples (n=4/sample) were purified and quantified using Qubit HS and/ or BR assay before they were sent off to the Milner centre for Evolution at University of Bath for Illumina Miseq sequencing service. The sequence data was reported back in FASTAQ files format. Each pair-end reads file was assigned in EzBioCloud 16S pipeline to conduct the 16S microbiome taxonomic of each sample (MTP) (http://www.ezbiocloud.net/.). The EzBioCloud 16S pipeline is interest-free for academic users and straight forward to analysing the 16S metagenomics data of a given sample, and it works as follow:

Initially, each row Illumina paired-end sequence reads (FASTAQ file) was first uploaded from Dropbox and or my computer browser, and the two sequences are merged by the overlapping sequence information. Primers used in PCR and the developing low-quality

reads were trimmed. Next, the pre-treated reads were then subjected to Quality controlled 16S reads which extracted non-redundant reads, excluding chimera, picking OTUs using open reference approach with 97% cut-off. Lastly, alpha diversity, rarefaction curve and overall sequence similarity index or values were calculated for the resulting microbiome taxonomic profile using USEARCH program (Yoon et al., 2017). Given a name for each microbiome sample (MTP unit) which represents each specific metagenomic sample that includes all information regarding reads, QC, number of identified species in the sample, thus MTPs can be grouped for respective comparison of different microbiome samples. The up-to-date PKSSU4.0 version was chosen to carry out the analysis of the 16S pair-end sequence outputs. The output of per-base sequence quality score (QC) of forward read of a representative sample (MT20) is demonstrated and supported in Figure 2.2.

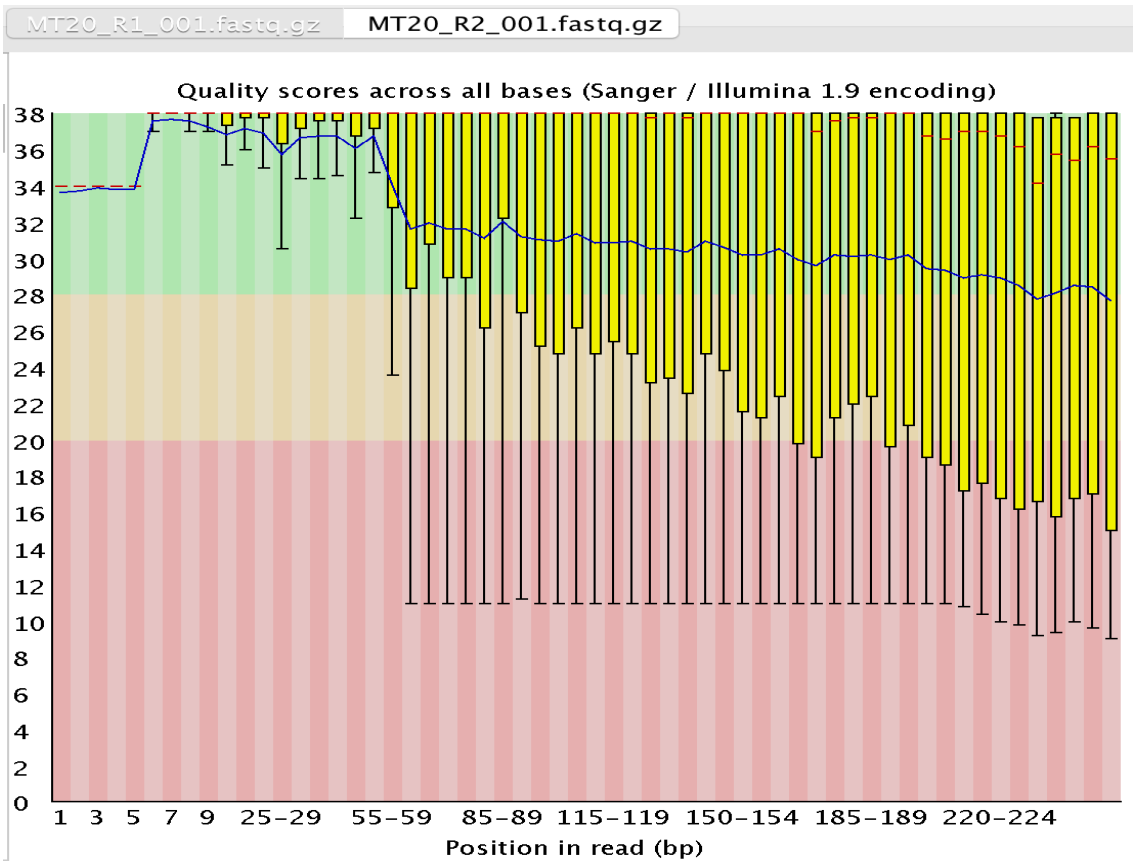


Figure 2.2: Representative output of per-base sequence quality report generated by Fast QC, shown here for the forward reads of Sample MT20. The median quality score for these reads is >30, indicative of high-quality sequence data.

2.11 16S Nanopore sequencing technology (OXFORD NANOPORE)

2.11.1 Optimisation of PCR.

The ZymoBiomics mock microbial DNA standard (100ng/µl) was used to investigate the effect of the amount of gDNA used as template on the yield of the PCR product and the resulting microbial community profile produced. 1µl of the gDNA was transferred into a 0.2ml thin-wall DNA-free PCR tube containing 9ul of sterile nuclease-free water. The gDNA was serial diluted to obtain 10, 100- and 1000-fold dilutions. This generated three

different amounts of gDNA template (46.6ng, 18.3ng, 2.24ng respectively). Each DNA template was amplified using a unique 16S nanopore barcoded primer sequence (27f and 1492r) that includes 5' tags to facilitate ligase-free attachment of rapid sequencing adapters in the respective multiplexing sample tubes, see Tables 2.9 and 2.10.

Invitrogen™ Platinum™ Hot Start PCR 2X Master Mix (Thermo Fisher, UK) was used as described in Table 2.9.

Table 2.9. PCR reaction tube used for 16S nanopore.

Reagent	Volume (µl)	Amount of gDNA template	Final concentration
2X Master Mix PCR	25	-	1X*
16S barcoded primer	10	-	-
DNA template	1	variable	<100ng/ run
Nuclease free water	Up to 50	-	-

Table 2.10. PCR condition used for 16S nanopore.

Steps		**Temperature** (°C)	**Time (minutes)**
Initial denaturation		95.00	1
25 cycles	Denaturation	95.00	0.30 s
	Annealing	55.00	00:30 s
	Extension	65.0	00:30 s
Final Extension		65.0	05.00
Hold		4.0	-

2.11.2 The library preparation of 16S amplicon used for nanopore sequencing

10 µl of each clean up PCR product was pooled into a 1.5 ml DNA Lo-Bind Eppendorf tube, the total pool was 30 µl. This volume was concentrated down to 10 µl as the final sequencing library is recommended, using ProNEX (Promega) magnetic beads. An equal volume of beads (30 µl) was pipetting into the pooled samples, mixed by flicking, and incubated at room temperature on a Hula-Mixer (Invitrogen) for 5 minutes. This time allows the pooled DNA library to bind to the magnetic beads. Then the tube was placed on magnetic rack to pellet magnetic beads and supernatant was discarded. The bead pellets were washed tow times by 200µl of fresh 70% ethanol, while the tube was still on the magnetic rack. The beads were briefly centrifuged, re-pelleted by magnetic rack and the remaining ethanol was discarded. The bead-pellets were left to dry for 1 minute, then taken off from the magnetic rack and pipetting into 10µl of 10mM Tris-HCl pH 8.0 and 50mM NaCl as recommended by the nanopore kit protocol. This was then incubated at room temperature for 2 minutes to elute the DNA back to the solution from the beads. The tube was briefly centrifuged, beads were re-pelleted by magnetic rack, and the supernatant was moved to 1.5ml DNA Lo-Bind fresh tube. The supernatant (pooled sequencing library) was taken immediately to the last library preparation and flow-cell loading step as recommended by the protocol SQK-16S024.

The prepared library was filled into an R9.4 RevD flow cell (FLO-MIN 106) with 900 available pores. The sequencing ran for 48 hours on a MinIT sequencer using MinKnow software, with exciting Guppy flipflop (FAST) base-calling (at this step the run was stopped for unknown reason for 5 hours). After 18 hours, an additional 200µl of running

buffer (FB) was added into the flow-cell through the priming port in order to increase the overall yield and quality score (QC). The sequencing run was completed after 48 hours, and the outcome of raw FASTA5 files was uploaded from the MinIT sequencer to my college Dr Natalie Ring' CLIMB account through a LaCie 3TB external hard drive.

2.11.3 The analysis of 16S nanopore sequence data

The FASTA5 files were base called on CLIMB and demultiplexed by using Guppy flipflop (FAST) and the command as follow:

guppy_basecaller --input_path [path/to/fast5/folder] --save_path [path/to/output/folder] --config dna_r9.4.1_450bps_fast.cfg --barcode-kits SQK-16S024 --num_callers 4 --cpu_threads_per_caller 4

Each FASTA5 read was base called by Guppy to produce FASTQ reads that were subsequently assigned to barcode bins, based on barcode sequence Guppy distinguished on the read. Because of the relatively high errors rate (i.e., r9.4, mainly ~10% error) some reads were assigned to the mismatch barcode bin, possibly read can assign to a barcode that did not use during the sequencing, to the unclassified barcode bin which was used during the sequencing but with the read did not derived from, or the correct barcode. While for other kit barcodes, the originated errors of demultiplexing step can be decreased by two distinct software tools (Deebinner and Guppy) that only hold on to the reads for which the two tools were agreed on a barcode. However, the 16S Barcoding kit 1-24 (SQK-16S024) is still recently developed by OXFORD NANAOPORE technology and based on our knowledge the Guppy is the only software can recognise its barcodes. In addition, the

barcode bins have multiple FASTAQ file each of which has approximately 4000 reads. The multiple FASTAQ files linked into single FASTAQ files, a single for each barcode using Ubuntu or "Cat" command and a "for" loop see below:

"for barcode in barcode01 barcode02 barcode03 barcode04 barcode05 barcode06 barcode07 barcode08 barcode09 barcode10 barcode11 barcode12 barcode13 barcode14 barcode15 barcode16 barcode17 barcode18 barcode19 barcode20 barcode21 barcode22 barcode23 barcode24 unclassified; do cd /path/to/barcode/bin; seqtk seq -a $barcode.fastq > $barcode.fasta; done"

Lastly, the 16S microbiome taxonomic profile (MTP) of each sample was conducted using EPI2ME WIMP pipeline. The first step in this workflow is to determine the quality score as well as the rapid real-time species identification and quantification for long sequence reads (how many reads/sample). However, the pipeline is interest-free, but the access of it is limited for users. While only with a few clicks all information regarding classification at different taxonomic levels (e.g., Phylum, genus and species), % relative abundance taxa, data distribution can be visualised via built in graphical manipulatable graphs (see figure 4.3 in chapter 4).

2.12 Statistical Analysis of the data obtained from bacteria-free *M. sexta* larvae growth and development

Two-way ANOVA statistical tests was used to determine the significance of differences between groups of larvae at different larval stages, using Prism Graph pad software v.8. The mean of the body mass (g) of larvae within each study group or population was compared with that of typical larvae were fed on typical colony diet/rearing condition.

3.Chapter 3 The impact of antibiotic on the gut microbiome population of *M. sexta* using a culture-dependent approach

3.1 Overview of the *M. sexta* gut microbiome.

The gut microbiome composition across vertebrates and invertebrates is extensively varied with more than 1000 phenotypes in humans, several hundred in termites, and a few tens in Lepidoptera, while there is an almost complete absence of a resident gut population in aphids. Lepidopterans are composed of one of the most diverse insect orders (Daniel E Shumer, *et al*., 2017; Voirol., *et al*., 2018). However, there is no clear evidence of the functional role of a resident gut microbiome in these caterpillars. A study by Hammer et al. (2017) reported that caterpillars harbour little or no resident gut bacteria compared to other insect orders. This was reported as being due to the alkaline conditions in the gut, making it challenging for resident gut bacteria to thrive. However, other studies have demonstrated that Lepidopterans do indeed possess a resident gut microbiome, which plays an essential role in acquiring critical nutrients and aids in the digestion of complex carbohydrates and proteins, strengthening the host immune system and aiding the host to overcome plant anti-herbivore defences. In a literature review conducted by Voirol et al. (2018) on the type and diversity of the bacterial symbionts in Lepidoptera, the majority of the resident gut microbiome that was identified belonged to the *Proteobacterium* phylum (42%) and *Alpha* and *Gamma-proteobacteria* classes being the most predominant (72%). Bacteria from the *Enterobacteriaceae*, *Bacillaceae*, *Pseudomonadaceae*, *Staphylococcaceae*, and *Enterococcaceae*, families were present (>60%). In addition, significant variability in the gut microbiome is observed across Lepidopterans, with the

resident gut microbiome in species such as *Manduca sexta* not being elucidated and identified. Hammer et al. (2017) conducted a study wherein they attempted to characterize the gut microbiome of wild-leaf caterpillars using 16S rRNA gene sequencing and quantitative PCR. Wild-type *Manduca sexta* showed a reduced bacteria load of approximately 61,000-fold compared to resident gut microbiome of other animals. Indeed, bacteria-free larvae of *Manduca sexta* has been generated by the latter author and the rearing of *Manduca sexta* larvae underwent germ-free conditions. The process that was utilised to obtain bacteria-free *Manduca sexta* larvae were as follows: newly hatched larvae were treated with 0 to 1.68mg of antibiotics per millimetre dissolved in distilled water and reared in separate conditions. To render the food sterile, leaves were sprayed with water in which antibiotics were dissolved in and allowed to dry before the larvae were fed with the sterilised food. The antibiotics that were used to generate sterile larvae and sterile food were rifampicin, tetracycline and streptomycin that were dissolved in a 1:2: 4 ratios. The suppressive antibiotic treatment used to sterile the gut bacteria of *Manduca sexta* led to a 14-365-fold reduction in the resident gut microbiome of *M. sexta* larvae. Despite that the sequencing of 16S rRNA of the faecal samples from larvae fed with these leaves that were sprayed with antibiotics was still shown the presence of leaf-associated bacteria such as *Staphylococcus, Escherichia, Enterococcus* and *Sphingomonas* thus demonstrating that the majority of the resident gut bacteria species were food-derived (Hammer *et al.*, 2017). However, the type of resident gut microbiome in *Manduca sexta* was not clearly characterised in this study.

3.2 Aim of this study

The aim of first study was to investigate the effect of the tetracycline supplementation of the Bath colony standard food on the gut microbiome population of *M. sexta* using a culture-dependent method.

3.3 Objectives

The objectives of this study were as follows: (1) Rearing *Manduca sexta* with different diets (antibiotic supplemented food and antibiotic-free food) and to determine the effect of diet on the microbial population, (2) to determine the effect of different exposures time to antibiotics on the microflora of the larvae, (3) to identify suitable growth conditions of the bacteria in terms of media, temperature and oxygen requirement, (4), to enumerate, isolate and identify the gut microbiome population by macroscopic characterization and 16S rRNA gene sequencing based PCR.

3.4 Results

3.4.1 Characterisation of the *M. sexta* gut microbiota by direct culture.

It was attempted to characterise the gut microbiota of *M. sexta* by directly plating bacteria from the gut contents onto different bacterial culture agar plates, followed by incubation under either aerobic or anaerobic conditions.

The University of Bath *Manduca* colony is reared by feeding on food supplemented with tetracycline to prevent the introduction of bacterial pathogens into the colony. It was unknown if the use of tetracycline would suppress or modify the bacterial gut flora. Thus, experiments were performed to investigate the gut microbiota of *M. sexta* both with and without supplementation of the food with tetracycline.

In each experiment, five groups of larvae were reared under the following conditions.

G1: standard conditions: tetracycline supplemented food throughout rearing.

G2: tetracycline-free food throughout rearing.

G3: tetracycline-supplemented food for the first three days, tetracycline-free food after that. G4: tetracycline-supplemented food for the first seven days, tetracycline-free food after that. G5: tetracycline-supplemented food for the first ten days, tetracycline-free food after that.

The weight of the late 5th instar larvae (15days) of each different diet system group (n=5/group) was measured and recorded prior to harvesting the gut composition from each group of larvae (see figure 3.1). Once the larvae reached the end of the 5^{th} instar stage of development (day 15), the gut contents were dissected, resuspended in PBS and aliquots plated onto five different agars to enable the growth of a wide range of different bacteria:

Nutrient agar (NA) – a general agar for the growth of a wide range of non-fastidious bacteria.

Brain heart infusion agar (BHI) – recommended for the growth of fastidious bacteria, including *Streptococci.*

Blood agar (BA) – a rich medium that promotes the growth of fastidious bacteria including *Streptococci* and *Haemophilus.*

MacConkey's agar (MA) – a selective and differential agar for the growth of gram-negative rods, particularly *Enterobacteriacea* and *Pseudomonas.*

De Man, Rogosa and Sharpe agar (MRS) – selective agar for the growth of lactic acid bacteria, including *Lactobacillus, Streptococcus, Pediococcus* and *Leuconostoc.*

Two different dilutions of the gut content suspension were plated, and the resulting colonies were counted, and colony characteristics were noted. Mostly, the numbers of colonies recovered on each agar showed great variation between larvae of the same group. Generally, very low numbers of bacteria were recovered by plating of resuspended gut contents directly on to agar plates. Bacteria were recovered on NA, BHI and BA (non-selective agars) and MacConkey and MRS (selective agars) suggesting that a number of different bacteria were recovered. The variety of colony morphologies observed on the agars supported this (Table 3.3 and see below). A summary of the number of various colony morphologies observed on agars and recovered from larvae that underwent different diet and rearing conditions in G1-G5 is shown in Table 3.1**,** 3.2, 3.3 and 3.4**.**

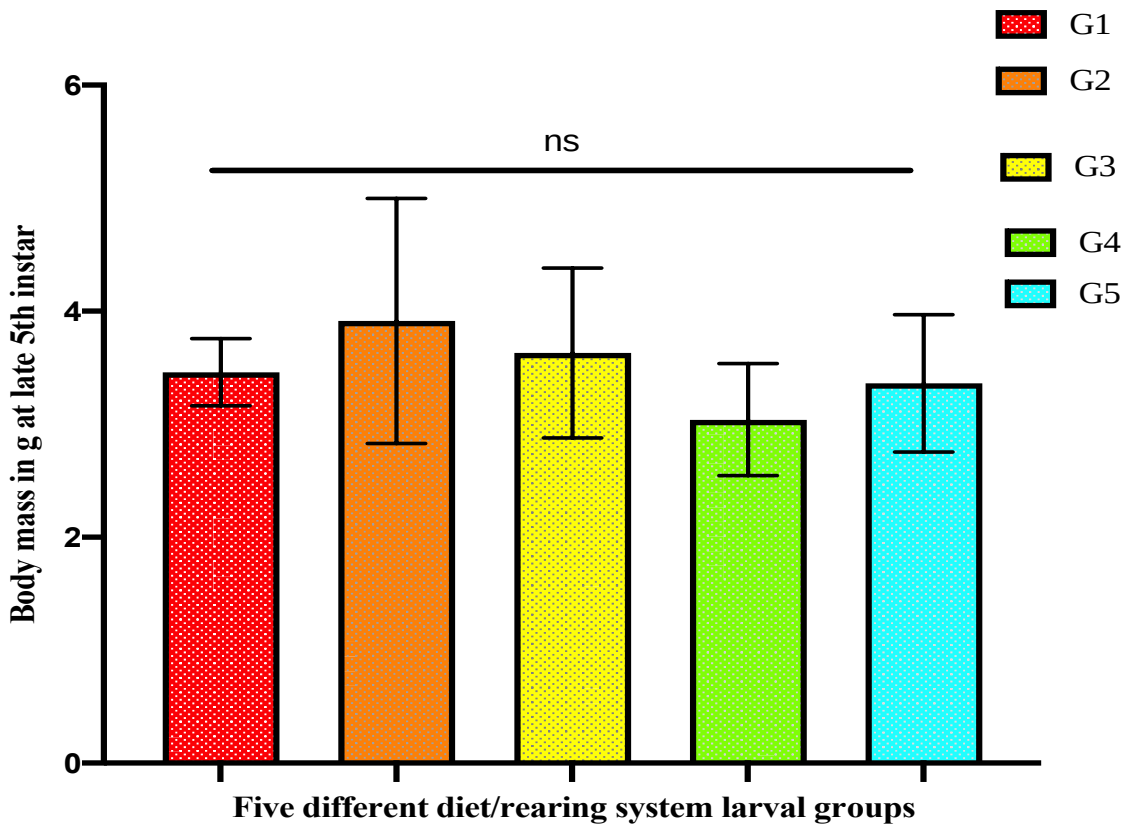


Figure 3.1. The average body weight at late 5th instar larvae day 15 (n=5/group) in each different diet/rearing system group. One-way ANOVA test (Prism V.8) was used to determine whether the use of antibiotic with different exposure times will affect the larval body weight. Hence, no statistically significant differences were observed between five different diet groups (denoted ns, $P < 0.05$, SD of G1, 2, 3, 4 and 5 are 0.266, 0.969, 0.672, 0.443 and 0.543 respectively).

No significant changes in the weight of the larvae fed under conditions of five different rearing systems were observed. Larvae reared with tetracycline free food (G1) displayed the highest mass, followed by larvae reared with tetracycline supplemented fed for three days and tetracycline free food after three days (G3). Larvae reared with tetracycline-supplemented food for the first seven days (G4), tetracycline-free food after that showed slightly lower weight compared to other groups (ns, $P > 0.05$).

Table 3.1 The number of colonies recovered from five different agar/larvae groups using the 1:2 dilution of gut content (n=5/group).

	5th instar Larvae number	1:2(v/v)				
		Nutrient agar	MacConkey agar	Blood agar	De Man Rogosa & Sharp	Brain Heart Infusion
G1	1	0	2	3	0	0
	2	>100	0	12	0	1
	3	>100	0	10	0	>100
	4	>100	0	>100	0	>100
	5	>100	0	3	1	>100
G2	1	0	0	0	1	0
	2	25	0	1	1	0
	3	1	1	0	0	1
	4	40	9	2	0	2
	5	1	1	0	0	1
G3	1	1	1	3	3	2
	2	0	0	9	1	0
	3	>100	0	1	>100	2
	4	1	110	23	>100	>100
	5	>100	0	1	>100	1
G4	1	1	>100	1	>100	150
	2	0	18	0	52	51
	3	0	51	1	98	116
	4	2	39	1	84	51
	5	1	2	0	3	>100
G5	1	0	82	1	83	89
	2	1	58	4	121	130
	3	1	39	1	112	64
	4	0	2	3	3	0
	5	0	10	0	4	7

Table 3.2 The number of colonies recovered from five different agar/larvae groups using the 1:20 dilution of gut content (n=5/group).

	5th instar larvae number	1:20 (v/v)				
		Nutrient agar	MacConkey agar	Blood agar	De Man Rogosa & Sharp	Brain Heart Infusion
G1	1	0	0	1	0	0
	2	0	0	0	0	0
	3	0	0	0	0	4
	4	0	0	>100	1	0
	5	4	0	0	0	0
G2	1	0	1	0	0	>100
	2	1	0	0	1	0
	3	1	0	0	0	0
	4	1	0	>100	0	0
	5	1	0	0	0	0
G3	1	1	0	1	0	0
	2	0	0	0	0	0
	3	>100	>100	>100	100	>100
	4	0	29	25	37	15
	5	>100	0	1	100	>100
G4	1	0	33	31	65	23
	2	0	9	6	9	4
	3	2	8	12	12	11
	4	>100	9	8	11	3
	5	0	0	>100	0	1
G5	1	1	7	2	8	4
	2	0	14	13	27	17
	3	1	11	18	13	6
	4	0	1	1	1	0
	5	0	0	1	1	1

Table 3.3: Repeat experiment, the number of colonies recovered from five different agar/larvae groups using the 1:2 dilution of gut content (n=5/group).

	Group number	1:2(v/v)				
		Nutrient agar	MacConkey agar	Blood agar	De Man Rogosa & Sharp	Brain Heart Infusion
G1	1	>100	>100	100	1	>100
	2	>100	4	3	0	83
	3	>100	>100	0	2	1
	4	>100	1	29	2	3
	5	0	>100	100	3	>100
G2	1	3	>100	6	3	4
	2	6	1	1	3	2
	3	>100	>100	>100	>100	>100
	4	3	6	4	1	4
	5	0	1	38	1	2
G3	1	0	4	>100	1	>100
	2	1	0	0	0	7
	3	0	1	0	2	2
	4	5	2	1	1	3
	5	56	0	>100	0	1
G4	1	0	0	0	1	2
	2	0	0	0	0	2
	3	2	1	0	1	2
	4	2	0	1	0	6
	5	4	1	3	0	91
G5	1	1	4	103	0	3
	2	0	1	1	1	31
	3	0	1	1	0	1
	4	3	0	1	1	1
	5	3	3	3	3	3

Table 3.4: Repeat experiment, the number of colonies recovered from five different agar/larvae groups using the 1:20 (v/v) dilution of gut content, (n=5/group).

	Group number	1:20 (v/v)				
		Nutrient agar	MacConkey agar	Blood agar	De Man Rogosa & Sharp	Brain Heart Infusion
G1	1	>99	42	59	0	50
	2	1	1	>100	0	2
	3	0	1	0	0	0
	4	3	2	0	1	7
	5	>100	>100	>100	0	>100
G2	1	0	1	0	0	>100
	2	1	0	0	0	1
	3	1	>100	>100	>100	>100
	4	1	1	1	0	1
	5	0	0	3	0	1
G3	1	0	7	1	1	0
	2	0	2	0	0	32
	3	0	0	0	0	0
	4	0	1	0	0	4
	5	0	3	0	1	>100
G4	1	0	1	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
	4	0	0	1	0	6
	5	0	0	1	0	4
G5	1	0	0	1	0	>100
	2	0	0	1	0	>100
	3	1	0	2	0	0
	4	0	0	0	1	0
	5	1	0	>100	0	0

However, there was no obvious effect of diluting the gut content samples (n=5/group) on the number of colonies recovered. The more dilute suspension (1:20 v/v) did not appear to contain fewer bacteria than the more concentrated suspension (1:2 v/v). Interestingly, tetracycline presence in the food did not seem significantly affect the numbers or types of bacteria recovered. However, if recovery is inconsistent, this cannot be stated with certainty. The experiment was repeated twice. In one repeat, the larvae were grown for an extra two days to produce a greater volume of gut fluid, and in the other repeat, a wider range of dilutions of gut content suspensions was used. However, the same variation between larvae was observed. The lack of correlation between the level of dilution of gut content and the number of colonies observed suggested that simple resuspension of gut content in PBS did not allow for efficient and reproducible sampling of gut bacteria. The gut contents contained a lot of solid matter that made pipetting of the contents difficult. Attempts were made to better homogenise the gut contents in order to release gut bacteria from particulate matter and produce a homogenous suspension. Gut contents were homogenised by vortexing with 1mm sterilised glass beads before plating, but this did not produce greater consistency of results. Two different diet based larval groups were studied. G1 represent 1^{st} instar larvae that were reared on standard conditions: tetracycline supplemented food throughout rearing. While G2 1^{st} instar larvae were fed with tetracycline-free food throughout rearing until they became 5^{th} instar larvae at day 15 respectively (n=3/group, see Table 3.5, 3.6, and 3.7).

Table 3.5: The number of colonies observed on different agar media for G1 larvae that were fed with antibiotic supplemented food, and G2 larvae fed without antibiotic throughout rearing time (15 days), using 1:10 dilution of the gut content samples that had been homogenised using 1mm glass beads (n=3/group).

Group	1:10(v/v) dilution				
	NA (aerobes)	BA (aerobes)	Mac (anaerobes)	BHI (aerobes)	MRS (anaerobes)
G1, 1	0	0	0	1 (Large &Creamy)	0
2	2 (Large &Creamy)	1 (Small &Creamy)	0	0	0
3	0	7 (Small &White)	0	2 (Unorganized)	0
G2, 1	0	0	1 (Mucoid)	0	0
2	0	0	1 (Unorganized) 1 (mucoid)	0	
3	0	1 (Small &White)	0	2 (Large &Creamy)	2 (Small &Creamy)

Table 3.6: The number of colonies observed on different agar media for G1 larvae fed with antibiotic-supplemented and G2 larvae fed on antibiotic-free food throughout rearing time, using 1:100 dilution of the gut content samples that had been homogenised using 1mm glass beads (n=3/group).

Group/larvae	1:100(v/v) dilution				
	NA (aerobes)	BA (aerobes)	Mac (anaerobes)	BHI (aerobes)	MRS (anaerobes)
G1, 1	0	0	0	0	0
2	0	0	0	0	1 (Large &Creamy)
3	0	0	0	0	1 (unorganised)
G2, 1	0	2 (Small &Creamy)	0	1 (colourless)	0
2	0	0	0	0	0
3	0	0	0	12 (Small &Creamy)	0

Table 3.7: The number of colonies observed on different agar media for G1 larvae were fed with antibiotic supplemented food, and G2 larvae were fed on antibiotic-free diet respectively, using 1:1000 dilution of the gut content samples that had been homogenised using 1mm glass beads (n=3/group).

Group/larvae	1:1000(v/v) dilution				
	NA (aerobes)	BA (aerobes)	Mac (anaerobes)	BHI (aerobes)	MRS (anaerobes)
G1, 1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	0	0	0
G2, 1	0	0	1 (mucoid)	0	0
2	0	0	0	8 (Small &Yellow)	0
3	0	0	0	2 (colourless)	0

3.4.2 Diversity of bacteria recovered from gut contents

Representatives of each colony type recovered on each agar were isolated and screened for their macroscopic characteristics. This revealed variable colony characteristics in terms of morphology, colour, and size, as seen in Table 3.8. Up to twelve different colony types were recovered from the five different larval groups. As many as nine different colony

morphologies were recovered from a single group. Larval group 3 exhibited the most diverse colony morphologies, including nine different types recorded as large and creamy, small and creamy, large and white, small and white, small and pink, unorganised, mucoid, colourless, and confluent. Larval group 2 (fed with an antibiotic-free diet for 13 days) revealed the lowest diversity with five types observed in larval group 3 fed with an antibiotic diet for three days then switched to antibiotic-free food for the remaining period (13 days). In larval group 1, seven different colonies were recovered, with the highest percentage of large and creamy, small and creamy, small, and white colonies. Larval groups 4 and 5 shared a similar number of eight different types of colonies, including large and creamy, small and creamy, large and white, small and white, small and pink, unorganised, mucoid and confluent. The ‘small and creamy’ colony type was most frequently recovered from all groups across experiments. Such basic phenotypic characteristics indicate bacterial diversity but do not allow the identification of the recovered bacteria.

Table 3.8: The Isolates investigated and their phenotypic (colony morphology) characteristics.

Bacteria	Origin/ Experiment	Origin/ Group	Origin/ Media	Isolation Date	Phenotypic characteristics
MS1	2	2	NA	16/08/16	Confluent
MS2	1	1	NA	10/06/16 16/09/16*****	Small, Creamy
MS3	2	2	NA	16//08/16	Unorganized,
MS4	2	3	NA	16/08/16	Large, Creamy
MS5	1	1	BA	10/06/16	Large, Creamy
MS6	2	2	BA	16/08/16	unorganized (filamentous)
MS7	2	2	MRS	16/08/16	Large, Creamy
MS8	2	4	BHI	16/08/16	Mucoid
MS9	2	2	BHI	16/08/16	Mucoid
MS10	2	4	MRS	16/08/16	Large, White
MS11	1	5	MRS	16/06/16	Large, Yellow
MS12	1	1	BHI	10/06/16	Small, White
MS13	2	2	BA	10/08/16	Unorganized
MS14	2	1	BA	16/08/16	Small, Creamy
MS15	1	1	MRS	16/06/16	Small, Yellow
MS16	1	3	BHI	16/06/16	Colourless
MS17	2	1	BA	16/08/16	Large, Cream
MS18	2	4	MRS	16/08/16	Small, White
MS19	2	2	BA	16/08/16	Large, White
MS20	2	2	BHI	16/08/16	Mucoid
MS21	2	3	BHI	16/08/16 16/09/16*****	Small, White
MS22	2	1	MA	16/08/16	Small, Pink
MS23	2	1	MA	16/08/16	Large, Pink

* Re-isolated

BA: blood agar, NA: nutrient agar, BHI: brain-heart infusion agar, MRS: De Man, Rogosa and Sharpe agar, MA: MacConkey agar.

3.4.3 Identification of the bacteria by 16S rRNA gene sequencing

Genotyping can reveal the precise identity of microorganisms, and various techniques are used for this purpose. The methods can derive from culture-dependent or culture-independent processes. One commonly used approach is the Sanger sequencing of the 16S rRNA gene of bacteria (Yarza et al., 2014; Yoon *et al.*, 2017). The 21 bacterial isolates detailed in Table 3.9 and Figure 3.2 were identified by amplifying and sequencing their 16S rRNA gene, using two different sequence databases for analysis. As seen in Table 3.9, the sequence analysis using the BLAST tool provided information about the isolates at genus and species level. Still, a clear identity was not defined as closely related bacteria exhibited the same percentage of sequence similarity and could not be distinguished. Indeed, sequence analysis using the EZ-taxon tool was more discriminatory and allowed better differentiation between closely related species. This is because the EZ-taxon database contains only sequences of type strains of bacteria and better identification of them was determined (Chun et al., 2007). Sequencing of the 16S rRNA gene allowed the identification of seven genera, namely: *Bacillus* (*B*), *Lysinibacillus* (*L*), *Viridibacillus* (*V*), *Oceanobacillus* (*O*), *Pseudomonas* (*P*), *Staphylococcus* (*S*) and *Lactobacillus* (*Lb*) and 11 groups of species including *B. subtilis*, *B. licheniformis*, *B. aerophilus*, *B. cereus*, *Lysinibacillus pakistanensis, L. sphaericus, V. arenosi, O. massilliensis, P. monteilii, S. haemolyticus* and *Lb. casei*. Most of the species were well discriminated using the EZ-taxon tool except the *B. cereus, B. aerophilus, B. subtilis* and *Lb. casei* isolates that exhibited the same percentage similarity with closely related species and thus could not be clearly identified (e.g., *B. cereus*: *B. thuringienisis* and *B. anthracis*; *B aerophilus*: *B. altitudinis* and *B. stratosphericus*; *B. subtilis*: *B. tequilensis*; *Lb casei*: *Lb paracasei*). The

percentage identity between the query sequence and the reference sequence was above 99% except for isolate MS7. It was 97.69 % similarity with the 16S gene from *O. massilliensis,* displaying 17 base pair differences indicating a potential new bacterial species. In general, the dominant genera and species were spore-forming bacteria initially classified in the *Bacillus* genus. *Lysinibacillus, Viridibacillus,* and *Oceanobacillus* are separate genera but are very closely linked to the *Bacillus* genus. Microorganisms belonging to the latter are Gram, catalase, and oxidase-positive, endospore-forming, rod-shaped bacteria. Most bacteria included in such genera grow aerobically, but some are facultative anaerobe.

These results demonstrate diversity in the microbiota of the gut of *M sexta* at the genus and species level. This diversity was a group (diet) and agar dependant, although some species were similar to different groups and agars. However, the number of isolates screened is too limited to define the real impact of antibiotic within the food on the diversity of the species occurring in the larval gut. Hence, the bodyweight of late 5^{th} instar larvae within each group showed no statistically significant difference (ns, $P < 0.05$). Despite that, the use of antibiotics with different antibiotics exposure times did not affect the body weight of these larvae where they were individually fed on (see Figure3.1).

With regards to the methodology applied in this study, it was noticed that the sequencing of the 16S rRNA gene was not able to differentiate closely related bacteria in some cases. For example, MS1, MS3, MS14, MS22, and MS23 were recovered from different larval groups on different agars (NA, Mac, BHI) and had distinct colony morphology types (confluent, unorganised, small creamy, small pink, and large pink, respectively), see Tables 3.8 and 3.9**.** Surprisingly, all of these isolates were identified as *Lysinibacillus*

pakistanensis (identical type strain), with the same high level of confidence (>99% sequence similarity of the 16S rRNA gene between bacterial isolates). It is recognised that although very widely used, sequencing of 16S rRNA gene for identification of bacteria has limitations for discriminating closely related species and should be used in combination with other genotypic techniques for a better characterization of the microorganisms (De Clerck and De Vos, 2004; Wang et al., 2007; Yoon *et al.*, 2017).

The sequence derived from isolate MS7 showed 97.69% similarity to *O. massilliensis*, with 17 base pairs difference. This potentially indicates that the isolate represents a new species of bacteria, and advanced studies should characterize the isolate. Moreover, the use of culture-independent methods such as metagenomic methodologies was later considered in this thesis to detect and identify underrepresented species or those that are difficult to recover by cultivable techniques (Strathdee and Free, 2002). This can provide more reliable information about the diversity of gut microorganisms but currently is relatively expensive than Sanger 16S rDNA gene sequencing method, and the data is very complex to interpret.

In addition, Clustal Omega database was utilized to construct the evolutionary relationships between the sets of closely related bacteria found in the gut of *Manduca sexta*. FR12205830 (*Pseudomonas (P) plecoglossicida /P. monteilii/P. putida)* was closely related to FR12205840 (*P. plecoglossicida /P. Monteilii / P. putida*). FR12205846 (*Lb. casei /Lb. paracasei*) were observed to be closely related to FR12205844 (*Lactobacillus (Lb) casei /Lb paracasei*). FR12205835 (*Oceanobacillus (O) massilliensis/ O. polygoni*) was unique and did not have a close relationship with any of the other identified bacteria. Consequently, BLAST was used to compute a pairwise sequence alignment between sequence query of FR12205835 isolate and the database sequences using Neighbour

Joining method to calculate the distance between tow sequences with only higher scoring sequences is shown in a BLAST tree, see figure 3.4. FR122058547 (*Staphylococcus (S) haemolyticus / S.epidermidis*) was phylogenetically related to FR12205836 (*Viridibacillus (Vb) arenosi /Vb. Arvi*), FR12205843 (*L. sphaericus/ L. varians / L. mangiferihumi*), FR12205851 (*L. macroides/ L. xylanilyticus/ L. pakistanensis*), FR12205829 (*Lysinibacillus (L) macroides / L. pakistanenis/ Bacillus (B) fusiformis*), FR12205850(*L. macroides /B. velezensis/ L. pakistanensis*), FR12205842 (*L. macroides /L. xylanilyticus/L. pakistanensis*) and FR12205831 (*L. macroides /L. xylanilyticus /L. pakistanensis*), see Figure3.3. The analysis of the phylogenetic tree was based on the identification of 16S rRNA gene sequence similarity of the bacteria isolated from the gut of five different diet/agar of *M. sexta* larvae. The divergence of gene sequence between each closely related bacterial species (i.e., molecular clock-like-behaviour) was informative to infer their evolutionary relationships. Despite that the homologues 16S rRNA gene is highly conserved between different bacterial species and its length (~1500bp) is still enough to determine sequence variations (i.e., alignment) needed for phylogenetic information (Brown, J. W., no date, pp. 1-14).

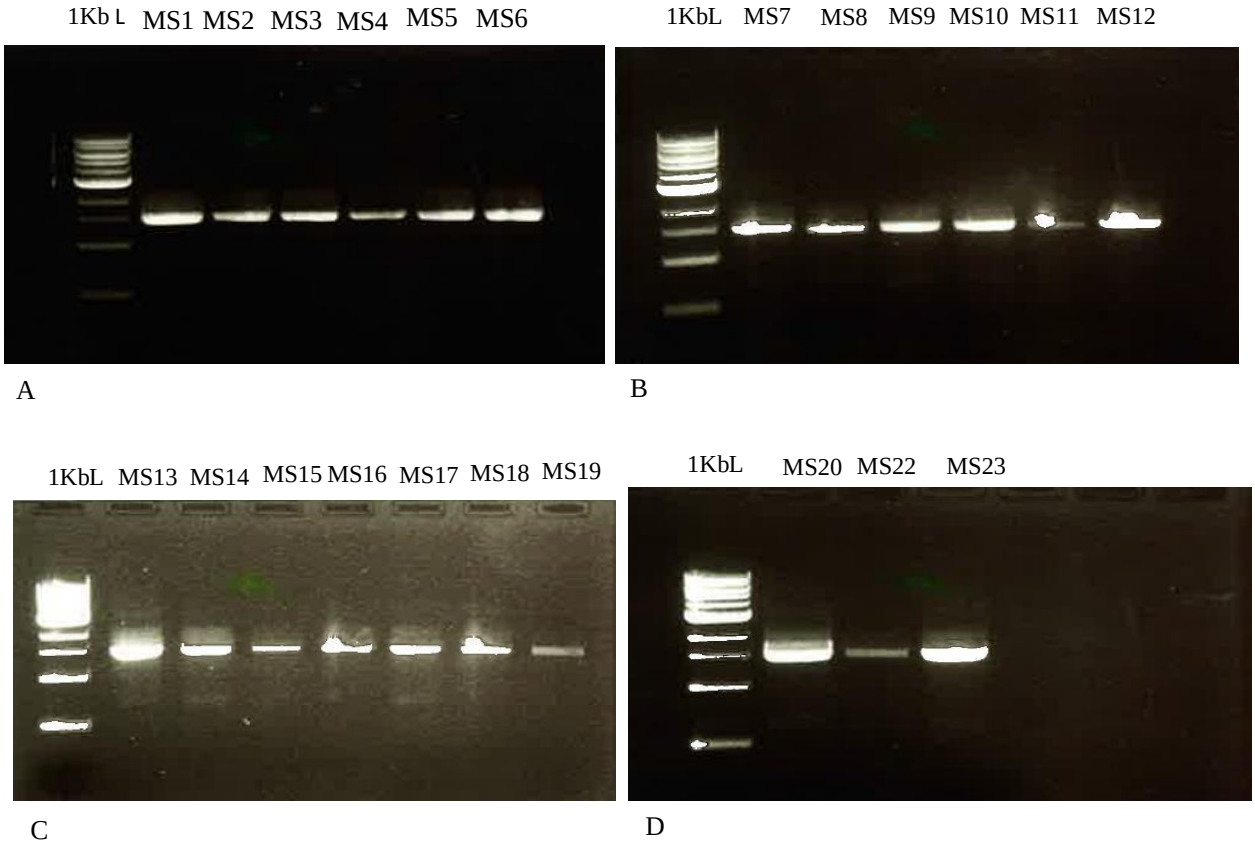


Figure 3.2: Positive PCR products of the amplification of the 16S rRNA gene of the bacteria isolated from the gut of *M. sexta* 5th instar larvae. The first lane of each 1% agarose TAE gel image represents the 1Kb DNA marker used to measure the expected molecular size ~1500bp of the 16S rDNA gene. The remaining lanes in A, B, C and D gel represent each annotated band corresponding to each bacterial isolate.

Figure 3.3: A screenshot of a constricted phylogenetic tree using Clustal Omega database represented the homology of the bacterial isolates as well the inferred evolutionary relationships between a set of those closely related species found inside the gut of *M. sexta* 5th instar larvae. (Code numbers refer to the corresponding identified isolates on Table 3.9).

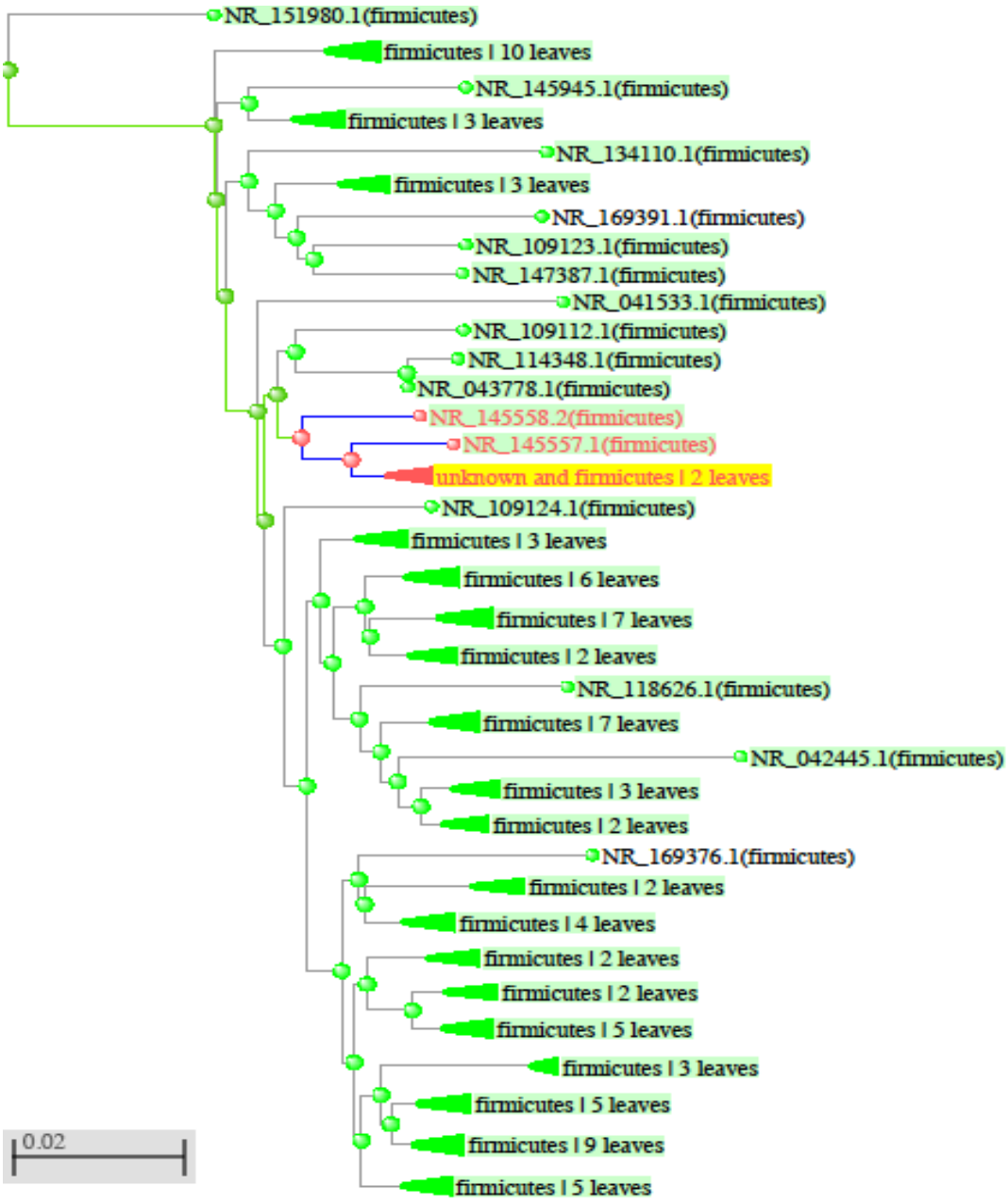


Figure 3.4: Shows a Blast tree was generated utilising BLAST to compute a pairwise local sequence alignment method between 16S rRNA gene sequence query (ID IcI/Query_26355) of MS7 isolate and database sequences. Neighbour Joining method was used to calculate the distance between two sequences (max sequence difference 0.75) and only the top hit scoring sequence is displayed in the tree. The MS7 isolate displayed 97.69% sequence similarity with *Oceanobacillus. massiliensis* sp. with 17 base pairs differences indicating to the new bacterium species.

Table 3.9: Identification of the bacteria by 16S rRNA gene sequencing combined with BLAST and EZ-taxon analyses.

Bacteria Code	Sequence code	16S rRNA gene sequencing/ BLAST identification		16S rRNA gene sequencing/ EZtaxon identification	
		Identity	Similarity (%)	*Identity*	Similarity (%)
MS1	FR12205829	*Lysinibacillus (L) macroides / L. pakistanenis/ Bacillus (B) fusiformis*	99	*L. pakistanensis*	99. 36
MS2	FR12205830	*Pseudomonas (P) plecoglossicida /P. monteilii/P. putida*	99	*P. monteilii*	99.78
MS3	FR12205831	*L. macroides /L. xylanilyticus /L. pakistanensis*	99	*L. pakistanensis*	99.49
MS4	FR12205832	*B. cereus /B. anthracis/ B. thuringiensis*	99	*B. anthracis /B. cereus*	100
MS5	FR12205833	*B. altitudinis /B. pumilus/ B. aerophilus*	99	*B. aerophilus / B.altitudinis / B. stratosphericus*	99.60
MS6	FR12205834	*B. licheniformis/B. sonorensis / B. aerius*	99	*B. licheniformis*	99.46
MS7	FR12205835	*Oceanobacillus (O) massilliensis/ O. polygoni*	98	*O. massilliensis*	97.69
MS8	FR12205836	*Viridibacillus (Vb) arenosi /Vb. arvi*	99	*Vb. arenosi*	99.74
MS9	FR12205837	*B. subtilis/ B. velezensis,/ B. methylotrophicus/ B. tequilensis*	99	*B. subtilis or B. tequilensis*	99.75 99.75
MS10	FR12205838	*B. thuringienisis /B. cereus / B. toyonensis*	99	*B. cereus/ B. thuringienisis / B. toyonensis*	99.44
MS11	FR12205839	*B. altitudinis /B. aerius/B. pumilus / B. aerophilus*	99	*B. aerophilus / B. altitudinis/ B. stratosphericus*	100
MS12	FR12205840	*P. plecoglossicida /P. Monteilii / P. putida*	100	*P. Monteilii*	99.34
MS13	FR12205841	*B. licheniformis /B. sonorensis/ B. aerius*	99	*B. licheniformis*	99.66
MS14	FR12205842	*L. macroides /L. xylanilyticus/L. pakistanensis*	99	*L. pakistanensis*	99.34
MS15	FR12205843	*L. sphaericus/ L. varians / L. mangiferihumi*	99	*L. sphaericus*	99.19
MS16	FR12205844	*Lactobacillus (Lb) casei /Lb paracasei*	99	*Lb. casei / Lb paracasei*	100
MS17	FR12205845	*Not determined*		*Not determined*	
MS18	FR12205846	*Lb. casei /Lb. paracasei*	100	*Lb. casei /Lb paracasei*	100
MS19	FR12205854 7	*Staphylococcus (S) haemolyticus / S.epidermidis*	99	*S. haemolyticus*	99.67
MS20	FR12205848	*B. tequilensis /B. subtilis/ B. methylotrophicus,*	99	*B. subtilis /B. tequilensis*	99.78
MS21	FR12205849	*Not determined*		*Not determined*	
MS22	FR12205850	*L. macroides /B. velezensis/ L. pakistanensis*	99	*L. pakistanensis*	99.09
MS23	FR12205851	*L. macroides/ L. xylanilyticus/ L. pakistanensis*	99	*L. pakistanensis*	99.67

3.5 Gut composition sample treatments

Further attempts were made to treat the larval gut content samples using different techniques, as described in method chapter 2, to improve the recovery of bacteria from the gut of larvae. The gut contents appeared to particulate to be disrupted by either vortexing or homogenising with glass beads. A bespoke homogeniser for use with 50ml conical tubes, comprising a conical shaped metal 'pestle' that could be attached to an electric drill for disrupting the autoclavable gut content samples and easy to disinfect using, e.g., alcohol, between samples, was explicitly designed for this purpose. This device produced a visibly well homogenised sample than conventional vortexing with or without glass beads, and much easier to pipette. Two different diet system larval groups were included in this experiment. G1 larvae were reared and fed on antibiotic-supplemented diet, while G2 larvae were fed on tetracycline-free food throughout (n=3/group). Yet, the recovery of gut bacteria from these samples was not improved compared to the previous methods, see Table 3.10 and 3.11.

It is possible that processing gut content samples caused damage to bacterial cells that prevented their recovery. Alternatively, the dissection of larvae may release antimicrobial factors, such as larval haemolymph, into the gut contents that kill gut bacteria (Bevins *et al*., 1999).

Table 3.10: The number of colonies observed on agar plates and recovered from G1 larvae (antibiotic-supplemented food) and G2 larvae (antibiotic-free food) whose gut content samples were treated using MP method (n=3/group).

Group/larvae	$1:10_{(v/v)}$ dilution			$1:100_{(v/v)}$ dilution		
	NA	BHI	MRS	NA	BHI	MRS
G1, 1	0	0	0	0	0	0
2	0	3 (Small & Creamy)	0	0	0	0
3	0	2 (unorganised)	0	1 (Small &White)	1 (unorganised)	0
G2, 1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0

Table 3.11: The number of colonies observed on agar plates and recovered from G1 larvae (with antibiotic-supplemented diet) and G2 larvae (antibiotic-free food) whose gut content samples were treated using MP technique, (n=3/group).

Group/larvae	1:10(v/v) dilution			1:100(v/v) dilution		
	NA	BHI	MRS	NA	BHI	MRS
G1, 1	0	0	0	0	0	0
2	2 (Large &White)	0	0	0	1 (unorganised)	0
3	0	2 (Small &Creamy)	0	0	0	0
G2, 1	0	0	0	0	0	0
2	0	3 (Small &Creamy)	0	0	0	0
3	0	1 (Large & White)	0	0	0	0

3.5.1 Spike-ins the larval gut content sample with a known number of 1XL blue *E. coli* used as a marker

To investigate either damage to bacteria, or the action of antimicrobial factors, during

dissection of gut contents and processing of samples, a known number of laboratory *E. coli* were used to 'spike' gut contents samples and their recovery by plating following processing was measured.

The CFU/ml of overnight cultures of *E. coli* XL1-blue was calculated by serially diluting them and plating 100ul aliquots onto LB agar and counting the resulting colonies (Table 6). A suspension of OD_{600}=1.0 contained on average, 1.2 $x10^9$ CFU/ml. To each of five gut content samples, 100µl of *E. coli* suspension was added (approximately 1.2 $x10^8$ bacteria). The samples were mixed by vortexing, serially diluted and 100µl aliquots plated onto LB agar plates. The resulting colonies were counted and the proportion of the bacteria that were added to the samples that were recovered was calculated (Table 3.12).

Table 3.12: The number of colonies grown from serial dilutions of overnight cultures of E. coli (3 independent suspensions). The bacterial number per 1 OD unit was calculated for each suspension and averaged.

Bacterial suspension	OD neat	Dilution			CFU/ml/OD
		10^{-6}	10^{-7}	10^{-8}	
1	4.37	215	50	9	$1.14X10^{9}$
2	7.84	Too many	101	7	$1.28X10^{9}$
3	13.9	Too many	162	17	$1.17X10^{9}$
Calculated CFU/ml	1.0				$1.2X10^{9}$

3.5.2 The proportional percentage of the marker bacterium

Table 3.13: The number of colonies of marker E. coli recovered from gut content samples, calculated as the percentage of the bacteria added to the samples (n=5).

Larvae number	Dilution of the gut sample plated	Number of colonies recovered	Calculated CFU/ml based on recovered colonies	Volume of the diluted gut sample (ml)	Calculated total bacteria in gut content sample	% of marker bacteria recovered
1	10^{-2}	174	$1.74X10^{5}$	12.23	$2.13x10^{6}$	1.77
	10^{-3}	26	$2.6X10^{5}$		$3.18X10^{6}$	2.65
2	10^{-2}	191	$1.91X10^{5}$	10.52	$2.01X10^{6}$	1.68
	10^{-3}	46	$4.6X10^{5}$		$4.6X10^{6}$	3.83
3	10^{-2}	216	$2.16X10^{5}$	13.04	$2.82X10^{6}$	2.35
	10^{-3}	31	$3.1X10^{5}$		$4.04X10^{6}$	3.36
4	10^{-2}	171	$1.71X10^{5}$	11.06	$1.89X10^{6}$	1.58
	10^{-3}	34	$3.4X10^{5}$		$3.76X10^{6}$	3.13
5	10^{-2}	195	$1.95X10^{5}$	11.33	$2.21X10^{6}$	1.84
	10^{-3}	31	$3.1X10^{5}$		$3.51X10^{6}$	2.92

Surprisingly, only ~1-3% of the *E. coli* added into samples were recovered by plating on agar. This suggested that the gut content samples (n=5 individuals) contained factors that inhibited recovery of the marker bacterium or antibacterial factor(s) (e.g., haemolymph) that killed marker bacteria. Hence, the high pH (~8-9) of the *M. sexta* gut is often described as being harsh for bacteria to persist in (Allen et al., 2009; Hammer et al., 2017). However,

in this experiment the bacteria were exposed to the gut contents for a relatively short time (~15 minutes), and then the gut content was serially diluted with PBS very soon after the marker bacteria were added.

3.6 Chapter summary and discussion

Importantly, using culture-based methodology with limited conditions may allow only a subset of bacteria within the gut to be recovered. When studying diversity, both culture-dependent and culture-independent methodologies should be combined to obtain a robust estimate of microbiome diversity (Yashiro et al., 2011). However, until further studies are conducted, it can be temporarily said that Group 2 that included larvae fed without antibiotics exhibited the most diverse microbial profile. This is not surprising as no growth-limiting factor (tetracycline) was added to the diet. Species such as *L. pakistanensis, L. sphaericus*, and *P. monteilii* might not be affected by the presence of tetracycline, as they were recovered from group 1 larvae that were fed only a tetracycline-supplemented diet. On the other hand, some species recovered in group 2 but not group 1, such as *B. subtilis* and *B. licheniformis* may be more susceptible to the antibiotic. The positive or adverse impact of diet and the presence of antibiotics on the occurrence of specific bacteria in various environments are well documented (Ley et al., 2008; Van Der Hoeven, Betrabet and Forst, 2008; Colman et al., 2012). This study shows that this also applies to variable antibiotic exposure times as the resistance or susceptibility of bacteria to an antibiotic is dependent on the type of microorganisms. Jernberg et al. (2010) reviewed the short and long-term impact of antibiotics on the human intestinal microbiome and showed that ecological disturbances occur in the microbial composition after antibiotic administration.

It is worth mentioning that the defined and specific composition of the foods used to breed the larvae and that contained wheatgerm, sucrose, dried active yeast, Wesson's salt, Cholesterol, Sorbic acid, Choline chloride, and Methylparaben may have contributed to designing the microflora profile of the larvae (Ahmed et al., 1989). Previous studies that have investigated the microbiome of *M. sexta* gut also revealed the presence of numerous species belonging to genera such as *Bacillus, Staphylococcus, Pediococcus, Enterococcus, Citrobacter, Corynebacterium, Micrococcus, Paenibacillus, proteobacteria, Methylobacterium, Sphingomonas Acinetobacter, Enterobacter, Sphingomonas, Flavobacterium,* and *Delftia* (Brinkmann, Martens and Tebbe, 2008; Van Der Hoeven, Betrabet and Forst, 2008; Mason et al.,2011).

There are similarities and differences in the microbial profiles obtained in this study and previous investigations. The differences can be attributed to different factors, such as the origin of the *M. sexta* screened, the diet, the media used to isolate the bacteria, the incubation conditions, and techniques used for the identification of the bacteria. For example, in the studies of Brinkmann, Martens and Tebbe, (2008), PCR-single-strand conformation polymorphism (PCR-SSCP), reverse transcriptase (RT)-PCR-SSCP, and stable isotope probing (SIP) were used to detect and identify the bacteria. One major difference in this work is the dominance of spore-forming bacteria of the *Bacillus* group of genera. However, as mentioned earlier, a limited number of isolates were included in this study because of the use of only culture-dependent methods that narrow the diversity of microbial profile. The difference observed in this work between the gut microbial profile of larvae fed with an antibiotic added food and those fed with antibiotic-free food was also reported by Van Der Hoeven, Betrabet and Forst, (2008). The latter authors showed that in

insects on a regular diet (without antibiotic), mainly Gram-positive cocci such as *Staphylococcus* sp. and *Pediococcus* sp. are dominant. However, their number decreased significantly in insects fed with an antibiotic (kanamycin and streptomycin) supplemented food. The decrease in the number of genera and species was accompanied by an increase in other bacterial species such as *Methylobacterium, Sphingomonas, Acinetobacter,* and *Paenibacillus* species. Van Der Hoeven, Betrabet and Forst, (2008) explained the difference in the microbial community of the two types of insects because bacteria dominant in the antibiotic-fed larvae were resistant to the antibiotic.

In contrast, those who disappeared or decreased in number were more susceptible. These experiments revealed that the Bath *M. sexta* colony larvae contain a diversity of bacteria, assessed by their growth under aerobic and anaerobic conditions on different agar media. The microorganisms include mainly Gram-positive bacteria, but several Gram-negative bacteria were also detected. The predominant isolates were spore-forming bacteria belonging to the genera *Bacillus, Lysinibacillus, Enterococcus, Viridibacillus,* and *Oceanobacillus.* Other types of bacteria include *lactic acid bacteria, Staphylococci, and Gram-negative Pseudomonas.* The specific types of bacteria isolated from the gut of *M. sexta* reared under different conditions most definitely demonstrates the presence of bacteria belonging to the *Enterococcaceae, Bacillaceae, Pseudomonaceae, Staphylococcaceae,* and *Enterobacteriaceae.* These bacteria are the most widespread and have been shown to constitute more than 60% of Lepidopterans gut microbiome composition (Broderick *et al.,* 2004; Voirol et al., 2018). The persistence of some gut bacteria species in *Manduca sexta* demonstrates a conserved group of bacteria present in the gut of a significant number of Lepidopterans, which is irrespective of the diet. Various

studies have also shown significant variabilities in the gut microbiome of Lepidopterans (Broderick and Lemaitre, 2012). The gut microbiome diversity might be promoted by multiple environmental factors such as, the insect diet, the developmental stage, and the gut physiology of the host. In terms of the environment, studies on the different microbial communities in wild-type insects and laboratory-reared insects of the same species showed differences in the gut microbiome even if the insects were fed from the same host plants (Staudacher et al., 2016). In addition to diet and environment, the developmental stage might also play an essential role in the gut microbiome diversity. However, this is beyond the scope of this study because all specimens that were used in this study were larvae of *Manduca sexta* that were at the late 5th instar (13 or 15 days) of the development.

The predominant bacteria group identified from the gut microbiome of *Manduca sexta* reared under different laboratory conditions belong to the *Enterococcaceae, Bacillaceae, Pseudomonaceae, Staphylococcaceae,* and *Enterobacteriaceae* families. Several of these bacterial species might be implicated in maintaining the necessary hosts physiological conditions, such as immune-related functions and nutrient availability (McMillan and Adamo, 2020). Alternatively, they may act as bacterial pathogens towards the host organism. *B. anthracis /B. cereus, B. aerophilus / B.altitudinis / B. stratosphericus, B. subtilis or B. tequilensis, B. thuringienisis / B. toyonensis,* and *B. licheniformis* are Gram-positive bacteria with a low GC content. These Bacteria belong to the *Firmicutes* phylum are spore-forming bacteria with aerobic and facultative anaerobic properties (Ehling-Schulz, Lereclus, and Koehler, 2019). Some of these bacteria may be pathogenic in insects and have been observed to trigger an immune response at infectious sites (Tran and Ramarao, 2013). *L. pakistanensis* are rod-shaped,

aerobic, motile, and Gram-positive bacteria with spherical spores produced terminally in slightly bulging sporangia (Ahmed *et al.*, 2014).

Lb. casei / Lb. paracasei are Gram-positive, facultative heterofermentative non spore-forming bacteria that belong to the *Firmicutes* phylum and class *Bacilli.* These bacteria are found as part of the gut microbiome in humans and dairy products and plant materials (Maldonado Galdeano et al., 2019). *Lb. casei / Lb paracasei* have been observed to improve immune function, decrease oxidative stress, and increase mucosal immune responses (Hill et al., 2018). *Viridibacillus arenosi* species from the novel *Viridibacillus* genus are rod-shaped endospore-forming Gram-positive, bacteria that grow at pH 7 to 9. They are strictly aerobes, oxidase-negative, and catalase-positive microorganisms (Heyrman *et al.*, 2005; Albert *et al.*, 2007). However, their presence in insect hosts, specifically in moths, have not been reported. One isolate (*O. massilliensis*) exhibited 97.69% sequence similarity to characterized species, perhaps indicating a new species of *Oceanobacillus* genus (see figure 3.4). It is a Gram-positive and strictly aerobic, rod-shaped bacterium with a motile polar flagellum that grows in 0-12 % NaCl and at pH 7.5-9.5. This genus was first described by Lu, Nogi and Takami, 2001, and was reclassified as independent genus by Yumoto et al. (2005), (Yumoto et al., 2005; Lu et al., 2011). The genus of this bacterium is comprised of 12 recognized species and two subspecies. These bacteria have been isolated from the gut of freshwater insects, freshwater fish, algal mat, and healthy human faecal sample. These bacteria belong to the *Firmicutes* phylum and the *Bacillaceae* family (Le Page et al., 2016; Roux et al., 2013). However, there is a relative shortage of data on the specific characteristics of this bacteria. Studies are still being carried

out to determine the symbiotic role of this bacterial species in the gut microbiome of the host organism.

S. haemolyticus can be described as a Gram-positive spore-forming facultative anaerobic and non-motile bacterium which has been encountered in the gut of insects such as the *Centipede* (arthropods), the mosquito, and flies (Yadav et al., 2016). These bacteria have been found to be destroyed in the adult stage of blood-sucking insects and only persists in the larval stages (Grabowski and Klein, 2017). Some species of these bacteria are opportunistic commensals that have been found in plant nectar and other plant species, which are fed on by a variety of insects and moths (Anjum et al., 2018). In humans, *S. haemolyticus* plays an essential role in nutrient availability in the form of metabolizing gluten (Caminero *et al.*, 2014). However, the role of this bacterium in nutrient availability in insects and moths has not been studied. *P. Monteilii* is a Gram-negative rod-shaped motile bacterium that have been found in the midgut of the microbiome of different larval stages of the Asian Tiger Mosquito and the gut of the diamondback moth (*Plutella xylostella*) (Indiragandhi *et al.*, 2008; Yadav *et al.*, 2016). The diversity of the gut microbiome of *Manduca sexta* has shown the presence of highly pathogenic and opportunistic bacteria species. Only *Lb. casei / Lb paracasei* have been reported to have essential functions in the regulation of the innate immune system via cell surface pattern recognition receptors or by direct activation of lymphoid cells, particularly in humans (Cross, 2002). Additional studies are needed to determine the beneficial or harmful relationships of the other bacteria species identified in the host gut.

There was no clear difference between the bacteria recovered from larvae fed on antibiotic-supplemented food or antibiotic-free food. This might be due to the fluctuated and limited

number of isolates isolated from larval gut, using only culture-dependent method in this study. The sequencing of the 16S rRNA gene combined with BLAST analysis generated valuable information for genus and species identification but could not clarify the identity. The sequencing of the 16S rRNA gene combined with EZ-taxon analysis provided better identification of most of the isolates, but some could not be discriminated at the species level. It was not possible to reproducibly recover bacteria from the gut contents. There was no consistency between dilutions and between larvae, and often very low numbers of bacteria were recovered. Numerous treatments of gut content samples were trailed, but none gave consistent recovery of bacteria. Using a maker bacterium used to spike into samples, suggested antimicrobial factors such as haemolymph might be present or released into the harvested gut contents sample that may reduce the number and diversity of bacteria recovered using these techniques (Allen *et al.*, 2009).

4.Chapter 4 Optimisation of culture-dependent and culture-independent approaches for characterisation of *M. sexta* gut bacteria.

4.1 Overview of culture-dependent and culture-independent approaches used in studying microbiomes

The first step in studying the host microbiome mainly involves collecting microbial mass specimens that will be utilized to analyse the different types of bacterial populations present in the host gut. Samples of the gut microbiome are most commonly obtained from faeces if obtaining samples from the gut is not possible. Both culture-independent and culture-based techniques have been used to characterise gut microbiomes (Greub, 2012; Raymond et al., 2019).

4.2 Culture-independent approaches for the characterization of *M. sexta* gut bacteria.

Cculture-independent approaches currently being used to characterize the gut microbiome of a host organism include DNA based approaches such as 16S rRNA (prokaryotic gene marker), 18S rRNA (eukaryotic gene marker), and ITS (eukaryotic marker) sequencing**.** The 16S rRNA approach has been utilized for the identification of the gut microbiome population of various animals including *M. sexta*. The sequencing of 16S rRNA gene requires the amplification of full length (~1500bp) or just variable regions of the gene using universal primers with a unique barcoded sequence (multiplex). The species-specific variability of these V regions has permitted the identification and the establishment of the phylogenetic relationships between microbiome bacteria (Johnson et al., 2019). 16S rRNA gene profiling has several advantages, particularly the high resolution

and the cost-effectiveness of the approach to directly identify the gut microbiome population using the next generation sequencing technology (e.g., Illumina Miseq or Highseq), (Gutell, Larsen and Woese, 1994; Chen et al., 2019). It offers a rapid and potential detection of the entire microbiome diversity profile. Yet, particularly in cases of low biomass specimens, biases may arise during for instance sampling, PCR and the library preparation of 16S rRNA amplicon. Thus, it was suggested that the inclusion of blank controls and a mock microbial community during the latter process would account for the commonly derived environmental contaminant DNA (the 'kitome') which can be later excluded in the respective downstream analyses of the microbiome profile), (Kim et al., 2017; Eisenhofer et al. 2019).

4.3 Culture-dependent approaches for the characterization of *M. sexta* gut bacteria.

Culture dependent approaches used for the characterisation of the gut microbiome of the host organism involves the isolation of the gut bacterial population and growing these bacteria in specific culture media, under reliable environmental conditions that attempt to replicate the bacterial growth characteristics of these bacteria (Ito et al., 2019). Lau et al. (2016) has designed 66 culture conditions with 33 different culture media with supplements for anaerobic and aerobic conditions (Lau et al., 2016). Despite the advancement in different approaches of culture-dependent methods for the gut microbiome characterisation, several disadvantages to this method exist. Only a minor fraction of the gut microbiome can be cultured under such conditions, due to the fact that it is almost impossible to mimic the growth conditions of the entire bacteria population in the gut (Lagier et al., 2012). Regardless of these limitations, culture-dependent techniques may

still be important to consider in microbiome studies. For instance, describing newly discovered bacterial species using only derived sequence data (OTUs) obtained from the database might not fully describe the novel isolated organism. As such, description of a novel isolate requires at least two phenotypic characteristics (Christensen, 2018). Also, description of full microbiome diversity involves identification of low abundance species that may not be detected via culture-independent approach (Ito et al., 2019). Each approach has its own strengths and limitations. Thus, it has been concluded that a combination of them would permit a robust estimation and interpretation of a complex diversity of microbial community profile (Yashiro et al., 2011; Pandya et al., 2017).

4.4 Objectives

Even following homogenisation dilution, and direct plating of gut contents failed to give reproducible or consistent recovery of bacteria from the gut contents of *M. sexta* larvae. Thus, it was necessary to develop alternative approaches for assessing their gut bacteria. The use of enrichment broth culture of bacteria in gut contents was investigated, as was characterising them without any culture (culture-independent) and using 16S rRNA gene sequencing to identify the bacteria in samples.

4.5 Selecting the kit for genomic DNA (gDNA) extraction.

A number of commercially available kits are available for extraction of gDNA from complex samples for use in microbiome studies, in which the identity and relative abundance of different bacteria in samples is characterised (Fiedorová et al., 2019). To test differences in the efficiency of several different kits, 3 of the most widely used were tested for extraction of gDNA from a mock community, the ZymoBiomics mock microbial

community. This commercially available mock community sample comprises equal amounts of three-gram negative bacteria (*Pseudomonas aeruginosa*, *Salmonella enterica* and *Escherichia coli*), five difficult to lyse gram positive bacteria (*Listeria monocytogenes*, *Lactobacillus fermentum*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus subtilis*) and lower levels of 2 difficult to lyse yeasts (*Saccharomyces cerevisiae* and *Cryptococcus neoformans*), all of which are inactivated.

Aliquots of the mock community were processed using 3 different kits. There was a difference in the gDNA yield achieved with each kit, Table 4.1, with the Qiagen DNeasy soil power kit yielding the highest DNA concentration, although a larger volume of the mock community was used (to follow the kit instructions), with the ZymoBiomics DNA miniprep kit giving a very similar yield. The Qiagen kit was chosen for future use, as per sample it was cheaper than the ZymoBiomics kit and gave good recovery of DNA.

Table 4.1: Yield of gDNA obtained using different extraction kits on the ZymoBiomics mock microbial community.

gDNA extraction kit	Sample volume used (µl)	Yield of DNA (ng/µl)
QIAGEN DNeasy soil power kit	250	0.832
Blank control sample (PBS)	250	Below measurable level
ZymoBiomics DNA miniprep kit	200	0.664
Blank control sample (PBS)	200	Below measurable level
Pure Link Microbiome DNA Purification Kit	200	0.228
Blank control sample (PBS)	200	Below measurable level

The ability to amplify the 16S rRNA V4 region from each gDNA sample was tested. All three DNA templates produced products, with the level of product proportional to the amount of gDNA used as template, (see **Table 4.2**).

Table 4.2: The yield of PCR product for amplification of the 16S rRNA gene from each DNA template produced from the 3 gDNA extraction kits.

Extraction kit	Template volume used (µl)	Yield of PCR product (ng/µl)
QIAGEN Power soil pro	1.2	44.8
Blank control	2	Below measurable level
ZymoBiomics DNA miniprep kit	1.2	34.2
Blank control	2	Below measurable level
Pure Link Microbiome DNA Purification Kit	1.2	10.2
Blank control	2	Below measurable level

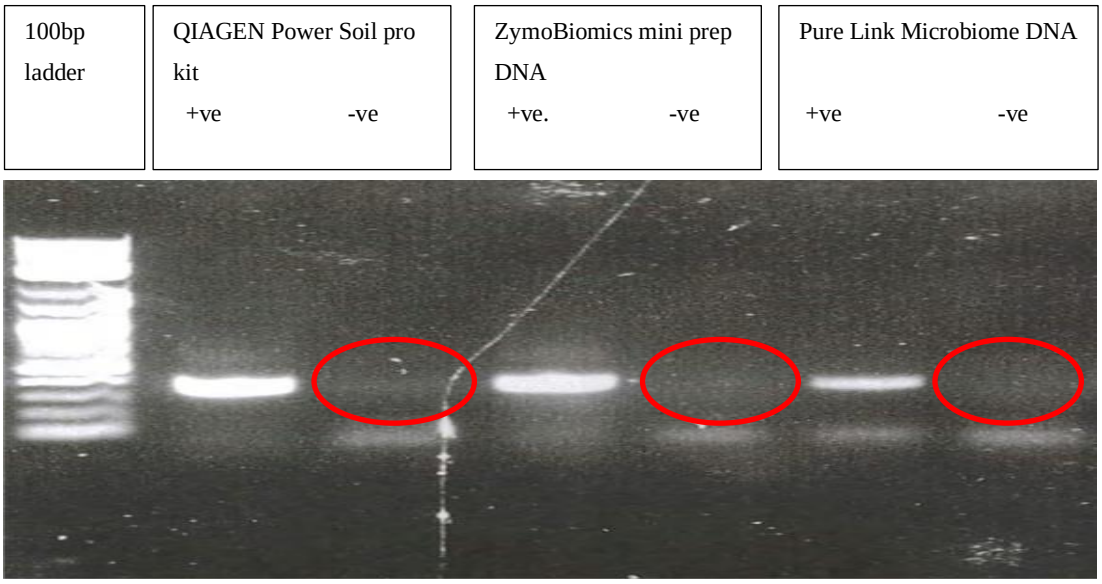


Figure 4.1: 1 % TEA gel image shows the amplification of the 16S V4 region of gDNA templates extracted using the 3 kits. The expected molecular size of each +ve PCR product of mock community/kit is ~252bp as they were measured using 100bp ladder. All 3 kits were produced effective gDNA templates for PCR. Feint bands (red circle) in the blank control lanes indicate that contaminant DNA carried through the gDNA extraction could act as a template for amplification of the V4 region but produced very low levels of product.

4.5.1 The impact of using different dilutions of gDNA template on the yield of the PCR product and Nanopore sequencing.

The amount of gDNA template used in the PCR to amplify the 16S gene can impact the sensitivity of detection of different members of microbiomes due to slightly different efficiency of PCRs by the different primer variants contained in degenerate primer samples (Fouhy et al., 2016; Kim et al., 2017). To investigate the effect of altering the amount of gDNA template on detection of the mock community members, the 16S gene was amplified from the ZymoBiomics mock microbial community DNA standard, using 3 different amounts of this template DNA (Kim et al., 2017; Fouhy et al., 2016).

A vast majority of microbiome analyses using 16S gene profiling have utilised short-read, Illumina DNA sequencing (Chen et al., 2019). There is great interest in the utility of the Nanopore platform, whose long-read sequencing enables the entire 16S gene, with all of the species information contained within it, to be sequenced in a single read (Johnson et al., 2019). To investigate the use of Nanopore sequencing, the amplicons produced from the three different template amounts were analysed by Nanopore. The Nanopore 16S barcoding kit (SQK-16S024) was used that contains universal 16S primers but with 24 different barcodes to allow multiplexing of samples in a single run. The yield of PCR product was dependent on the amount of template used (Figure 4.2), but equal amounts of product were used for Nanopore library preparation.

The PCR products (252bp) were purified and sequenced on the Nanopore MinIon platform, each of the three products were amplified with a different barcode (21, 22 and 23 from the Nanopore 16S Barcoding kit). This generated 1402820, 18250 and 595212 reads

respectively. It was not clear why relatively few reads were generated for the Barcode 22 product. Off the shelf pipelines for analysis of Nanopore 16S sequence data are limited.

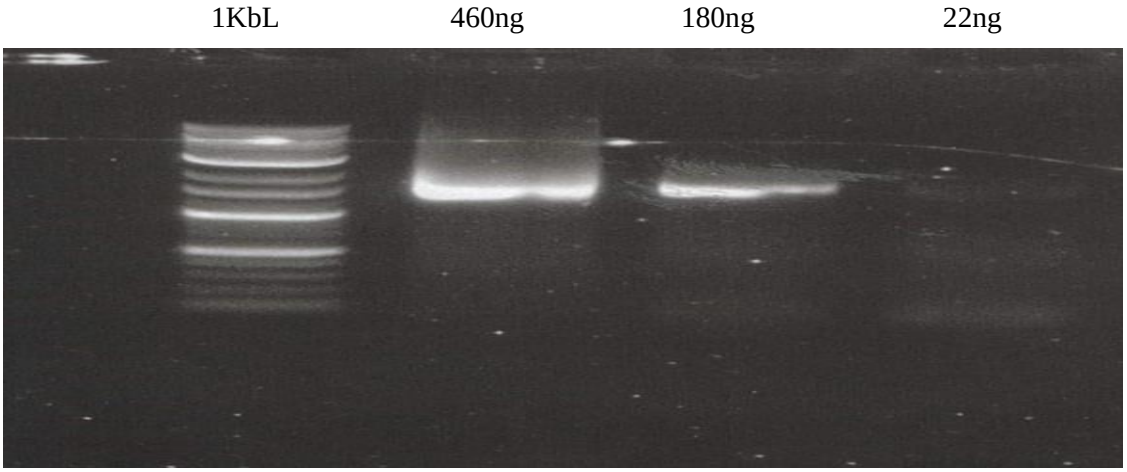


Figure 4.2. 1 % TEA agarose gel image of 16S PCR products amplified from three different dilutions of gDNA of the ZymoBiomic mock microbial community DNA standard (100ng/µl) using 16S 1-24 Nanopore barcoded primers kit. The expected molecular size of each amplicon is ~1500bp as measured using 1Kb ladder located in the first lane.

Those developed for use with short read sequencing often filter out most Nanopore derived reads due to the relatively low-quality scores that are associated with Nanopore sequence data. The sequence data generated here was analysed using the Nanopore Epi2Me 16S analysis workflow (www.nanoporetech.com). Currently, this web-based pipeline struggles with very large numbers of sequence reads. Thus, 35 000 reads were randomly subsampled for Barcodes 21 and 23, while all reads were uploaded for Barcode 22, and taxonomic profiling conducted. A very similar pattern was observed for all 3 samples. At the genus level, all 8 bacterial genera present in the mock community DNA standard were identified, but at unequal levels, with *Escherichia* and *Pseudomonas* generating relatively few reads,

and the pipeline appearing to misidentify some of the *Escherichia* reads as *Shigella*, which is very closely related to *Escherichia*, Figure 4.3. At the species level, the pipeline identified reads for *Bacillus*, *Listeria* and to some extent *Staphylococcus* as deriving from several species from each genus. This suggested that Nanopore based microbial 16S profiling is suited to identification of bacteria at the genus level, but may misidentify bacteria at the species level, particularly bacteria within genera that contain very closely related species (VanBraekel et al., 2020). In addition, the Nanopore 16S barcoding kit primers appear to have some bias in the evenness with which the 16S gene is amplified from different bacterial genomes in mixed samples. However, this is a common problem to processes that rely on degenerate primers amplify products from mixed samples (VanBraekel et al., 2020). On the plus side, Nanopore profiling was fast, with the entire process from PCR to generating sequence being completed in a day. The cost per sample, if maximum multiplexing is used is very comparable to the cost of Illumina sequencing, and the introduction of the small-scale Flongle flow cell promises to make Nanopore 16S profiling cheaper than most Illumina-based services (VanBraekel et al., 2020; Johnson, et al., 2019).

4.6 Variation in *Manduca sexta* gut microbiome composition

The work in chapter 3 showed that traditional plate-based culture could not consistently identify the bacteria within the *M. sexta* larvae gut. This could have been due to difficulty in recovering the bacteria from the gut content samples and/or variability in the gut bacteria present within the larvae guts in the Bath colony. Different approaches to characterisation of these bacteria were attempted.

4.6.1 Culture-free method

Differential centrifugation was used to try to isolate bacterial cells directly from gut content samples, and gDNA extracted directly from the resulting pellets. Pellets were recovered from pools of four 5th instar larvae at 15 days, Figure 4.4. Measurement of the concentration of the DNA produced by extracting these pellets revealed low yields, suggesting that in most cases the pellets contained low numbers of bacteria. The bacteria within the pellets were identified through 16S rRNA gene sequencing (see below).

Taxon	Cumulative Reads
Bacillus	5,896
Staphylococcus	4,754
Lactobacillus	3,885
Listeria	3,285
Salmonella	2,026
Enterococcus	1,866
Pseudomonas	458
Escherichia	435
Shigella	279
Citrobacter	128
Macrococcus	44
Enterobacter	39

Taxon	Cumulative Reads
Lactobacillus fermentum	**3,424**
Staphylococcus aureus	3,356
Salmonella enterica	2,018
Bacillus subtilis	1,846
Listeria welshimeri	1,606
Enterococcus faecalis	1,601
Bacillus mojavensis	1,167
Bacillus halotolerans	1,116
Listeria innocua	751
Staphylococcus simiae	434
Listeria seeligeri	373
Pseudomonas aeruginosa	363

Taxon	Cumulative Reads
Bacillus vallismortis	336
Escherichia fergusonii	325
Listeria monocytogenes	215
Bacillus nakamurai	180
Bacillus atrophaeus	178
Listeria ivanovii	175
Staphylococcus petrasii	161
Lactobacillus gastricus	144
Bacillus amyloliquefaciens	128
Staphylococcus haemolyticus	125
Shigella flexneri	123
Shigella sonnei	121

Taxon	Cumulative Reads
Bacillus	2,946
Staphylococcus	**2,451**
Listeria	1,780
Lactobacillus	1,593
Enterococcus	1,237
Salmonella	1,179
Shigella	108
Escherichia	103
Pseudomonas	98
Citrobacter	49
Enterobacter	24
Kosakonia	17

Taxon	Cumulative Reads
Staphylococcus aureus	2,106
Lactobacillus fermentum	**1,420**
Salmonella enterica	1,176
Listeria welshimeri	1,173
Enterococcus faecalis	1,123
Bacillus subtilis	1,006
Bacillus mojavensis	847
Bacillus halotolerans	577
Listeria innocua	386
Bacillus vallismortis	118
Listeria seeligeri	117
Bacillus atrophaeus	96

Pseudomonas aeruginosa	91
Lactobacillus gastricus	86
Staphylococcus capitis	81
Escherichia fergusonii	78
Shigella flexneri	70
Bacillus amyloliquefaciens	58
Listeria ivanovii	50
Staphylococcus simiae	42
Staphylococcus haemolyticus	37
Shigella sonnei	31
Listeria monocytogenes	29
Staphylococcus hominis	25

Taxon	Cumulative Reads
Bacillus	6,082
Staphylococcus	5,254
Lactobacillus	**4,038**
Listeria	3,985
Enterococcus	2,482
Salmonella	1,783
Pseudomonas	485
Escherichia	194
Shigella	163
Citrobacter	78
Enterobacter	35
Klebsiella	28

Taxon	Cumulative Reads
Staphylococcus aureus	4,553
Lactobacillus fermentum	3,541
Listeria welshimeri	2,609
Enterococcus faecalis	2,290
Bacillus subtilis	2,022
Salmonella enterica	1,782
Bacillus mojavensis	1,685
Bacillus halotolerans	1,292
Listeria innocua	823
Pseudomonas aeruginosa	447
Listeria seeligeri	301
Lactobacillus gastricus	253

Bacillus vallismortis	251
Bacillus atrophaeus	173
Staphylococcus capitis	157
Escherichia fergusonii	141
Staphylococcus simiae	122
Bacillus amyloliquefaciens	120
Listeria monocytogenes	115
Shigella flexneri	110
Listeria ivanovii	86
Staphylococcus haemolyticus	76
Staphylococcus hominis	55
Bacillus nakamurai	53

Figure 4.3: Microbial profiles of Barcode 21, 22 and 23 products as determined using the Nanopore Epi2Me 16S workflow. Left column, genus level identifications, Middle and Right columns: species level identifications for Barcode 21 (top row), Barcode 22 (middle row) and Barcode 23 (bottom row).

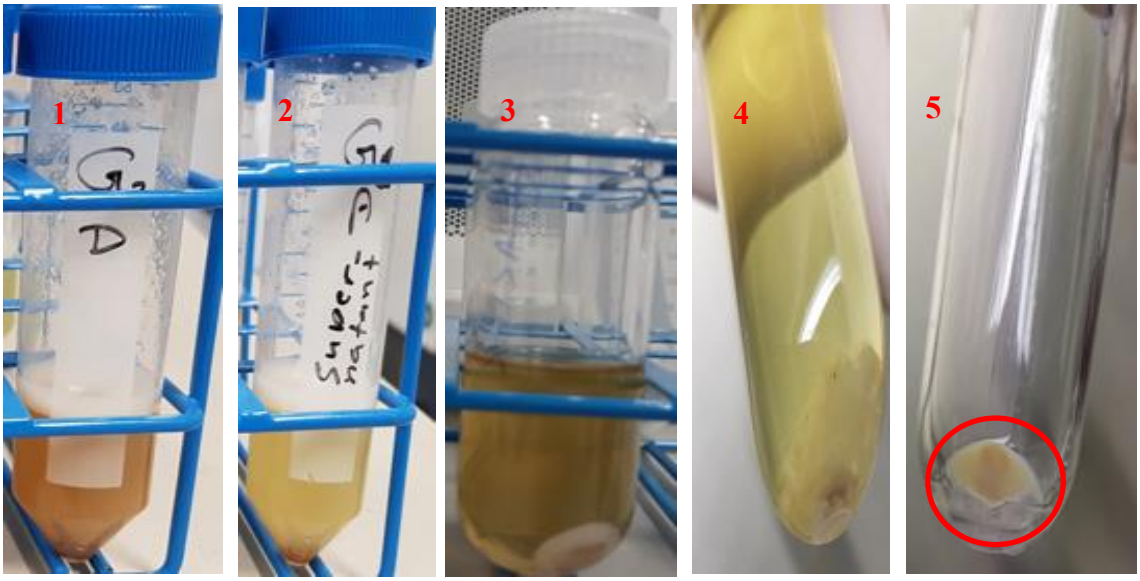


Figure 4.4: Photographs of the use of differential centrifugation to attempt to directly isolates bacterial cells from larvae gut contents. The pellet resulting when the supernatant remained clear was used for gDNA extraction using the Qiagen DNeasy soil power kit

4.5. Enrichment broth culture-dependent approach.

An alternative approach was to inoculate broth media with aliquots of gut content samples, and to culture bacteria present in the samples in these enrichment broths. This would permit bacteria present at low numbers in the gut contents to increase in number to help make their identification easier. However, enrichment broths represent different conditions to those in which the bacteria reside in the larvae guts and represent narrow ranges of growth conditions that may result in only subpopulations of the total bacteria present in the guts being able to grow (Lagier et al., 2012; Fraher, O'Toole and Quigley, 2012).

The OD_{600} of enrichment broth cultures (n=4/culture) was periodically checked every 24h (**Table 4.3**) at which points 100µl of each culture was plated onto the corresponding agar medium to further confirm the recovery of the bacteria. Usually, aerobic bacteria grow

faster than anaerobes and this was clearly seen in aerobic BHI broth cultures that were incubated for 1 to 3 days, while anaerobic TS broth cultures were incubated for 3 to 5 days. It was possible that bacteria growing in the anaerobic broths were either obligate or facultative anaerobes but were considered as anaerobic bacteria in this study. The increasing OD_{600} over time indicated bacterial growth in the cultures. In most cases, a single colony type was observed on the agar plates, suggesting a limited diversity of bacteria was recovered by this method (Greub, 2012; Raymond et al., 2019).

Table 4.3: The OD600 of enrichment broth cultures from a representative experiment. BHI- aerobic conditions, BHI broth. TS- anaerobic conditions, TS broth.

Sample Number	Medium	Time (hours)					
		0	24	48	72	96	120
1	BHI	0.005	0.011	0.020	0.120	0.190	-
	TS	0.001	0.001	0.096	0.109	0.160	0.185
2	BHI	0.010	0.025	0.128	-	-	-
	TS	0.025	0.214	0.510	-	-	-
3	BHI	0.012	0.176	0.209	-	-	-
	TS	0.001	0.001	0.010	0.012	0.108	0.197

4.7 Bacterial taxonomic profiles (MTPs)

Genomic-DNA samples were generated from pellets isolated directly from gut content samples (culture-free) and from enrichment broth cultures (enrichment broths) quantified using the Qubit system and submitted for sequencing at the Milner Centre Genomics Centre, University of Bath. 16S V4 region sequencing on an Illumina Miseq was performed. Data was returned as forward and reverse reads in FASTAQ files. The samples analysed and the sequence data generated are shown in Table 4.4. Unfortunately, sequence data was returned for only a portion of the total samples submitted for sequencing. Attempts to generate sequence data from all samples is on-going. A small number of samples generated a disproportionate number of reads (for example, MT36, Table 4.4.) while many samples failed to generate sufficient reads for analysis or reads with low quality scores.

Table 4.4: Samples (n=4/sample) for which sequence data was obtained.

Sample identification	Sample type	No. of reads generated
MT20	Day 15 larvae, BHI enrichment broth. Experiment 1	32 116
MT22	Day 15 larvae, culture-free. Experiment 1	30 504
MT30	Day 15 larvae, culture-free. Experiment 2	30 391
MT36	Day 15 larvae, TS enrichment broth. Experiment 2	3 398 136
MT40	Day 15 larvae. Culture-free. Experiment 3	31 304

The quality of the reads for each sample was assessed using Fast QC, (see Figure 2.2 in chapter 2**)**. Only samples that with sufficient numbers of reads (>10 000) and quality scores (Q>30) were taken forward for analyses.

4.7.1 Analysis using EzBiocloud 16S based MTP pipeline

The paired end read files were uploaded to the EzBiocloud server. The reads undergo pre-processing steps including merging paired-end reads, trimming primers used for PCR, filtering low quality reads to produce quality-controlled 16S reads (Chun et al., 2018). The

up-to-date database version (1.0) included in the EzBiocloud 16S microbiome pipeline was used for the analysis of the microbiome taxonomic profile (MTPs) of each sample. This included pulling out non-redundant reads, identification at different taxonomic levels via the EzBiocloud database, excluding chimeras, picking OTU counts by utilizing the open reference method with a 97% cut off. The last step in this secondary analysis is the calculation of alpha diversity (species richness) and refraction curves. The coupled processes generate the final microbiome taxonomic profiles that included all information of the MTPs. The microbiome taxonomic profiles of the samples are shown and displayed in Table 4.5, 4.6 and Figure 4.5.

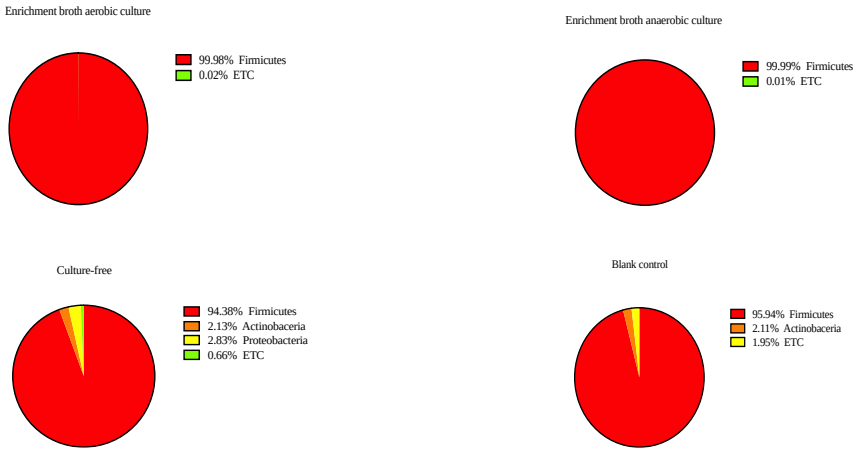
Table 4.5: Taxonomic profile of samples (n=4/sample) at the phylum level. Values are percentage relative abundance.

Sample/Phylum	Firmicutes	Actinobacteria	Proteobacteria	ETC
MT20	99.98	0	0	0.02
MT22	97.60	2.1	0	0.3
MT30	94.91	2.23	1.37	1.49
MT36	99.99	0	0	0.01
MT40	93.21	2.12	4.45	0.22
MT39 (Blank)	95.94	2.11	0	1.95

Table 4.6: Microbiome taxonomic profile of samples (n=4/sample) at the genus level. Values are percentage relative abundance.

Genus/Sample	MT20	MT36	MT22	MT30	MT40	MT39
	Enrichment aerobic	Enrichment anaerobic	Culture-free			Blank
Aneurinibacillus	96.91		11.30			
Bacillus	1.14			1.52		2.45
Bordetella					4.27	
Brevibacillus					6.77	
Clostridium					1.65	1.20
Clostridium g24		16.10		6.48		
Clostridium g34		43.28		17.10		
Enterococcus			2.69	2.04	2.23	1.61
Lactococcus			36.91	18.68	39.06	42.91
Leuconostoc					1.09	
Macrococcus			1.70		2.69	1.88
Paenibacillus	1.56	40.44		18.64		
Rothia			1.63	1.60	1.15	1.23
Staphylococcus				1.67	1.10	
Streptococcus			40.87	25.12	35.91	39.82
ETC	0.39	0.18	4.89	7.10		8.89

A



B

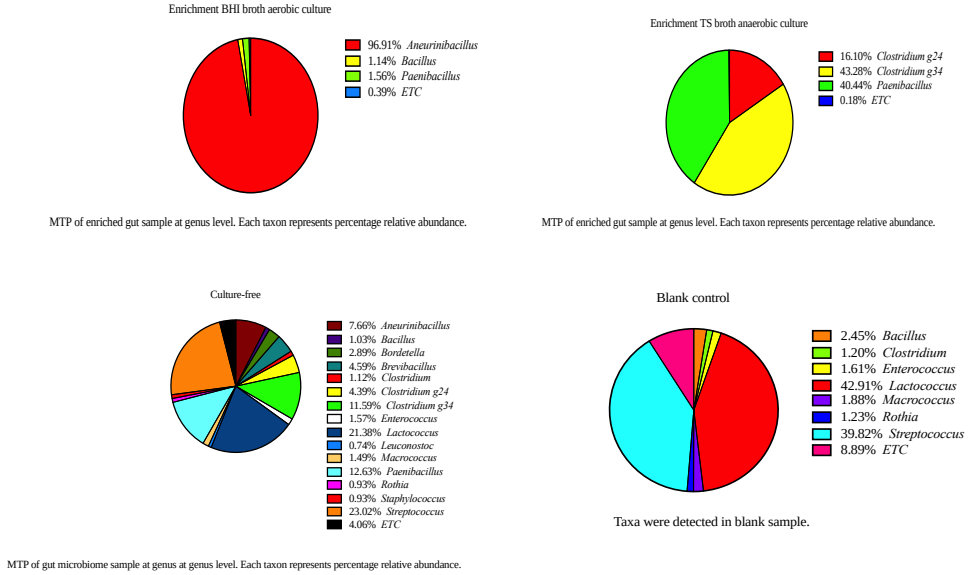
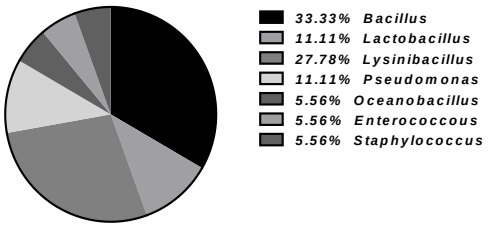
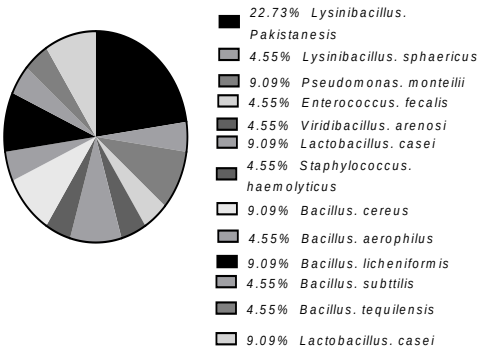


Figure 4.5: The microbiome taxonomic profiles of Bath colony at (A) the phylum and (B) genus level, using enrichment broth cultures and culture-free methods (n=4/sample). The sequencing of 16S rRNA V4 region using Illumina Miseq platform revealed a diversity of *M. sexta* larval gut microbiome down to the genus level illustrated by each pie chart. Percentage represents relative abundance of each taxon in the gut microbiome sample.



Microbial diversity profile at genus level obtained from culture-dependent method (Direct inoculation of agar media).



Microbial diversity profile at species level, culture-dependent (Direct inoculation of agar media).

Figure 4.6: Shows the microbial diversity profile at genus and species level using direct culture-based method. Percentage indicates to the predominant bacterial isolates were frequently isolated from different diet rearing systems of *M. sexta* larval groups (conducted in chapter 3). Sanger sequencing of 16S rRNA gene was used and the resulting FASTA Q files were assigned to EzBiocloud database to identify each isolate based on top hit 16S rDNA gene sequence similarity.

4.8 Discussion

4.8.1 Recovery of the gut microbiome of *M. sexta* through culture-dependent and culture-independent approaches

The first aim of this thesis was to characterise the gut microbiota of the Bath colony *M. sexta*. Initial attempts to recover the gut bacteria by direct plating onto several different agars results in the growth of low and inconsistent numbers of colonies. It was thought that this was due to the nature of the gut content samples, being viscous and containing a lot of particulate matter. Attempts were made to treat the gut content samples to make them more homogenous in order to free the bacterial cells into suspension. This produced no improvement in the number of bacteria recovered. A number of the isolates that were recovered were identified by Sanger sequencing of their 16S rRNA genes and revealed many were bacteria of the genus *Bacillus* and other genera closely related to *Bacillus* (see Figure 4.6).

Different approaches to recovery of the gut bacteria were attempted. Differential centrifugation of the gut content samples resulted in a cell pellet which would contain the bacterial cells. Extraction of gDNA from pellets yielded low amounts of DNA, suggesting that low levels of bacterial cells were present in the pellets, that still contained presumably non-cellular material. Aliquots of gut contents were inoculated into enrichment broths and cultured under both aerobic and anaerobic conditions. This resulted in the growth of bacteria, but these cultures contained low bacterial diversity.

The low bacterial diversity after *in vitro* culturing in enrichment broth might have been because the enrichment broths used in this experiment do not contain factors that might be important for the recovery of other gut microbiome species (Greub, 2012; Raymond et al.,

2019). This might suggest that the enrichment broth culture conditions used in the recovery of the gut microbiome of *M. sexta* do not recapitulate the growth conditions of a significant number of bacteria present in the gut microbiome of the host organism. This was thus one of the significant drawbacks of this study because the use of culture-dependent conditions usually does not permit the identification of the full diversity of the bacterial population present in the gut of *M. sexta* (Lagier et al., 2012). A technical difficulty encountered during the culture-free identification of the bacterial isolates was that the blank sample (M39) was positive for contamination with bacterial DNA present during e.g., sampling, PCR or library preparation of the 16S amplicon (Kim et al., 2017; Eisenhofer et al. 2019). This might lead to false-positive results, which does not accurately account for the bacterial isolates present in the larval gut. Enrichment BHI broth under aerobic conditions and TS broth under anaerobic conditions were utilized to enrich gut bacterial isolates in order to ease their identification in this study. In contrast, in chapter 3, direct inoculation of gut content on solid agars, in most cases did not permit a sustainable isolation of bacteria even following homogenisation and dilutions of gut content samples (Greub, 2012).

4.8.2 16S MTPs of the gut microbiome of *M. sexta* larvae via culture-free and enrichment broth culture-dependent methods

From comparing the microbial profiles at the genus level, it appears that the profiles of the 3 culture-free samples are similar to that of the blank control sample. These three samples yielded very low levels of gDNA, making them susceptible to contamination by environmentally derived DNA from reagents (Kim et al., 2017; Eisenhofer et al. 2019).

The bacteria present at higher abundances that are not identified in the blank control sample includes *Aneurinibacillus*, *Clostridium* of the g24 and g34 groups and *Paenibacillus.* The *Aneurinibacillus* dominated the aerobic enrichment broth sample, while the others were identified from the anaerobic enrichment broth. While this is consistent with the idea that while enrichment broth culture increases the number of bacteria from gut samples, only certain bacteria grow under these conditions; but does not rule out that these are the dominant bacteria within the larvae guts (Lagier et al., 2012; Fraher, O'Toole and Quigley, 2012).

At the species level, in MT20 *Aneurinibacillus aneurinilyticus* was the dominant species (96.91%) while in MT36 the *Clostridium* comprise *C. indolis* (43.2%), *C. g24* (15.67%) and *Paenibacillus* comprised *P. motobuensis* (28.64%) and *P. azoreducens* (11.70%).

Thus, the gut bacteria of the Bath colony *M. sexta* larvae that were identified were mainly *Aneurinibacillus*, *Clostridium* and *Paenibacillus.* However, the requirement to use enrichment broth culture to recover sufficient numbers of bacteria for identification makes definitive description of the larval gut microbiome very difficult (Lagier et al., 2012; Greub, G., 2012; Raymond et al., 2019).

4.8.3 Identification of the gut bacteria of the Bath colony' *M. sexta* larvae

Identification of bacteria using Illumina based sequencing of the V4 region of the 16S gene revealed that many of the bacteria identified in the cell pellet samples (culture-free) were also identified in blank controls, controlling for the presence of contaminant DNA in reagents. Such contamination is a common occurrence in microbiome studies of low-cell number samples (Kim et al., 2017; Eisenhofer et al. 2019). *Aneurinibacillus* dominated the

aerobic enrichment broth sample while *Clostridium* and *Paenibaciillus* were identified in the anerobic sample. *Aneurinibacillus* are environmental bacteria of the *Paenibacillaecae* family (Grady et al., 2016), suggesting that this family of bacteria are key components of the bacteria in the Bath colony. These were not identified in the blank controls and thus are residents of the Bath colony larvae guts. *Paenibacillus* are well-recognised environmental bacteria, often found associated with insects, including *P. larvae* that is the cause of foul-brood disease in honeybees. *Paenibacillus* produce a variety of carbohydrate degrading enzymes, and many also produce antimicrobial compounds (Genersch, 2010).

In chapter 3, of the identified bacteria, the predominant isolates were spore-forming bacteria belonging to the genera *Bacillus, Lysinibacillus, Enterococcus, Viridibacillus,* and *Oceanobacillus.* Other types of bacteria recovered less frequently included lactic acid bacteria, *Staphylococci,* and Gram-negative *Pseudomonas.* This was in contrast to the bacteria identified in this chapter, which were predominantly the *Aneurinibacillus, Bacillus, Clostridium, Clostridium g24, Clostridium g34, Enterococcus, Lactococcus, Staphylococci, Macrococcus, Rothia, Streptococcus, and Paenibacillus.* Thus, there were some similarities observed among bacteria that were isolated from the different larval groups in chapter 3 and 4; being those of the genera *Lactococcus, Staphylococci, Streptococcus,* and *Paenibacillus*. However, there were also differences in the bacterial composition described in chapter 3 and 4. In chapter 3, I observed a novel bacterium of the genus *Oceanobacillus,* which was not identified among the bacterial isolates in chapter 4. However, *Firmicutes* bacteria remained the most predominant phylum in all larval gut isolates observed across all samples. A technical

difficulty encountered during the identification of the specific genera of the bacteria by using Illumina Miseq platform sequencing 16S rRNA gene was due to the low depth of coverage of some of the sequencing runs in several bacterial isolates (Poretsky et al., 2014). Thus, this might account for the low number of identified bacteria from the gut isolates isolated from the different larval gut samples. A summary of a comparison between the gut microbiome diversity profiles of Bath colony were observed in chapter 3 and 4 is supported and demonstrated in Figure 4.5 and 4.6.

It is likely that the levels of bacteria in the larval guts are low, making recovery a problem. This is consistent with the findings of Hammer et al. (2017) who, during this project, reported that caterpillars do not contain a resident gut microbiota, with their gut bacteria derived from food-borne bacteria and that are transient within the gut. This report suggested that the bacteria within the caterpillar gut do not play a role in the caterpillar physiology, being only 'accidental' residents. This is very different to the situation for many other animals in which the gut microbiota is intricately involved in the animal's development and physiology. However, there will be a large number of bacteria in the caterpillar environment and on their food. It has been suggested that the low numbers of bacteria within the caterpillar gut is because of the gut environment. Caterpillars feed on plant material that contains antimicrobial compounds and often the pH of the gut lumen is very high, between pH 8-9, creating conditions in the guts that does not support the growth of many bacteria. However, the Bath colony of *M. sexta* is fed on an artificial diet and thus the guts of the larvae will not contain many of the plant-derived antimicrobial compounds, although the pH of the gut contents was observed to be alkaline, around pH8. The larvae are exposed to bacteria in their environment. Initially, the hatchlings eat the egg

components and will be maternally consumers of the egg-borne bacteria (Voirol et al., 2018). The eggs are laid in the adult moth chamber that will contain a high bacterial load. In the Bath colony, the eggs are disinfected before hatching, but this does not sterilise the eggs (see chapter 5) and viable bacteria remain on the egg surface. During growth, the larvae are exposed to bacteria in the *Manduca* growth room environment, and bacteria carried on the artificial diet food that is used. This is supplemented with tetracycline to help to protect the larvae from pathogens (Van Der Hoeven, Betrabet and Forst, 2008). Thus, it is not surprising that the bacteria identified in the guts of larvae are classed as environmental bacteria (Brinkmann, Martens and Tebbe, 2008; Hammer et al., 2017; Voirol et al., 2018). Interestingly, when a laboratory strain of *E. coli* was spiked into gut contents in order to investigate the efficiency of recovery, only a few percent of the bacteria were recovered. This is consistent with the presence of antibacterial factors in the gut contents, which could be host derived such as antimicrobial peptides and/or microbe derived (Allen et al., 2009).

These studies suggest that the Bath colony *M. sexta* caterpillar guts contain low numbers of mainly environmental bacteria.

5. Chapter 5 Rearing bacteria-free *Manduca sexta* as a microbiome research model

5.1 Overview on the development of Germ-free animal models

Various research studies have been conducted to determine the role of the gut microbiome in physiological processes such as digestion, the immune response, gut metabolism and the role of the microbiome in the modulation of the central nervous system (Dame, 1960; Baumann, Moran and Baumann, 1997). The use of germ-free animals to assess the impact of the gut microbiota on these physiological processes is of importance to study the interaction of the host and the gut microbiota. The term germ-free animal refers to an organism that is completely devoid of the presence of microbes such as bacteria, fungi, parasites and protozoa throughout the entire lifecycle of the animal (Dame, 1960; Baumann, Moran and Baumann, 1997). It is hypothesized that germ-free animals possess a sterile gut, devoid of microorganisms in embryo throughout the life stages. Fundamentally, to maintain these animals under germ-free conditions, neonates/larvae are frequently reared in sterile incubators and fed with artificial diets that are supplemented with or without antibiotics, to prevent the establishment of microbial communities that might be derived from environment and food sources (Engel and Moran, 2013). Examples of germ-free insects that have been successfully reared in the laboratory under sterile conditions are *Bombus terrestris* in which laboratory reared colonies were transplanted with faecal matter containing the gut microbial colonies that were resistant and susceptible to parasitic strains of *Crithidia bombi.* The host immune response to the transplant of the gut microbial community was measured using quantitative PCR technology. The

expression of six pro-immunogenic gene profiles was assessed in the germ-free colonies after transplanted of faecal material from the resistant and susceptible colonies. It was observed an increased expression of pro-immunogenic genes such as TEPA, defensin, *serpin27a*, *BGRP2* compared to the susceptible colonies wherein these genes were not highly expressed. The results of this study clearly demonstrating the ability of the insect gut to differentiate and regulate the host immune response towards invaders before the establishment of their own gut-microbiome (i.e., homeostais), (Näpflin and Schmid-Hempel, 2016).

In terms of the importance of the gut microbiome for the normal development of lepidopterans, a study was conducted by Habineza et al. (2019) to assess the effect of the manipulation of the gut microbiome of a common palm tree pest *Rhynchophorus ferrugineus* (Oliver) in altering the haemolymph nutrients availability. Indeed, the latter authors were utilized dechorionated eggs and the developing Germ-free larvae were subjected to feed on sterile artificial diet and maintain under sterile environment. Interestingly, a poor growth and development was observed in Germ-free larvae with a remarkable weight-loss comparing to that of control larvae. Surprisingly, exposing these Germ-free larvae to the gut microbiome obtained from conventional rearing larvae, particularly *Lactococcus lactis* and *Enterobacter cloacae* demonstrated a significant elevation of haemolymph nutrients levels was observed as well as that in the control larvae (phenotype). The findings of this study clearly demonstrate the role of gut microbiome on the maintenance of normal insect growth and development and highlight the insight of

using Germ-free insect model in studying the role lepidoptera gut microbiome (Habineza *et al.*, 2019).

5.2 Aims, objectives and approach.

This study aimed to devise a protocol for rearing bacteria-free *M. sexta* larvae, and to investigate the effect of the absence of bacteria on the growth and development of the larvae. The first question addressed in this study was as follows: “Is it possible to rear bacteria-free *M. sexta*?”. The objective of this study was to establish a bacteria-free insect model using the following approach: sterilizing eggs and maintaining eggs and larvae under bacteria-free conditions, either with or without a cocktail of broad-spectrum antibiotics. This involved creating sterile food for the caterpillars that are able to support their growth and development.

5.3 Results

5.3.1 The effect of bleaching eggs on hatching frequency.

Often, bleach is used to sterilise insect eggs for research studies. However, bleach will damage eggs and may affect their viability. The concentration of bleach and time of exposure of eggs to the bleach was optimised for both elimination of bacteria and egg viability. Nine different concentrations of the bleach were tested using different exposure times of 3, 5, 10 and 20 minutes for each concentration. Control groups of eggs were exposed to only sterile distilled water. The hatching frequency (HF) was the percentage of viable eggs in each group that had hatched at 4- and 5-days post-treatment (for small scale

experiments n=5-15/treated group). The eggs were incubated in bleach solution for the indicated time before washing by incubating in distilled water, see table 5.1

Table 5.1: Treatment with nine different concentrations of bleach and exposure times. However, treatment with higher concentrations of bleach resulted in very low hatching frequencies at all time points, with only sporadic eggs hatching.

$NaHClO^-$ %	Time (m)	Hatching				Contamination	HF %	HF %
		Post		Late				
		Survival	Total	Survival	Total		Post	Late
0.1	3	4	6	6	6	Yes	66.66	100
S.D.H_2O	3	4	10	10	10	Yes	40	60
0.1	5	1	5	5	5	Yes	20	100
S.D. H_2O	5	4	10	6	10	Yes	40	60
0.1	10	3	9	3	9	Yes	33.33	33.33
S.D.H_2O	10	7	13	7	13	Yes	53	53
0.1	15	0	11	0	11	Yes	0	0
S.D.H_2O	15	7	13	12	13	Yes	76.92	92.3
0.1	20	0	13	0	13	No	0	0
S.D.H_2O	20	7	14	13	14	Yes	50	92.85
0.2	3	0	6	2	6	No	0	33.33
0.2	5	0	6	0	6	No	0	0
0.2	10	0	11	0	11	No	0	0
0.2	15	0	6	0	6	No	0	0
0.2	20	0	13	0	13	No	0	0
0.25	3	0	6	3	6	No	0	50
0.25	5	0	6	0	6	No	0	0
0.25	10	0	6	0	6	No	0	0
0.25	15	0	11	0	11	No	0	0
0.25	20	0	11	0	11	No	0	0
0.3	3	1	6	1	6	No	16.66	16.66
0.3	5	0	9	1	9	No	0	11.11
0.3	10	0	11	0	11	No	0	0
0.3	15	0	15	0	15	No	0	0
0.3	20	0	10	0	10	No	0	0

$NaHClO^-$ %	Time (m)	Hatching				Contamination	HF%	HF%
		Post		Late			Post	Late
		Survival	Total	Survival	Total			
0.35	3	0	6	4	6	No	0	0.66
0.35	5	0	10	2	10	No	0	20
0.35	10	0	13	0	13	No	0	0
0.35	15	0	10	0	10	No	0	0
0.35	20	0	10	0	0	No	0	0
0.4	3	0	5	0	5	No	0	0
0.4	5	0	5	1	5	No	0	20
0.4	10	0	7	0	7	No	0	0
0.4	15	0	6	0	6	No	0	0
0.4	20	0	12	0	12	No	0	0
0.45	3	0	6	0	6	No	0	0
0.45	5	0	6	0	6	No	0	0
0.45	10	0	10	0	10	No	0	0
0.45	15	0	10	0	10	No	0	0
0.45	20	0	9	3	9	No	0	33.33
0.5	3	0	9	3	9	No	0	33.33
0.5	5	0	6	2	6	No	0	33.33
0.5	10	0	9	0	9	No	0	0
0.5	15	0	6	0	6	No	0	0
0.5	20	0	22	0	22	Yes	0	0
0.55	3	0	6	0	6	No	0	0
0.55	5	0	6	2	6	No	0	33.33
0.55	10	0	10	0	10	No	0	0
0.55	15	0	7	0	7	No	0	0
0.55	20	0	9	0	9	No	0	0

The treated eggs (n=5-15/treated groups) were placed on BHI agar and incubated at 26^{o}C for 5 days and the growth of bacterial colonies was monitored to gage whether the bleach treatment had sterilised the eggs. Considerable bacterial growth was observed on plates incubated with eggs treated with 0.1% bleach, demonstrating that this treatment was ineffective at sterilising the eggs.

No bacterial growth on BHI plates was observed from eggs treated with <, 0.55% or 0.6% bleach. To further check the sterility of eggs treated in this way, batches of 3 to 5 eggs were placed into both enrichment TS and BHI broth media using sterile forceps and incubated at 30^{o} C for 5days. Surprisingly, the OD_{600nm} of the broths with eggs treated with 0.55% bleach increased during the incubation period. Plating of 100µl of the growing broth cultures onto either BHI or TS agar confirmed the presence of contamination from the bleached eggs. Thus, sterilisation required high levels of bleach, but this decreased hatching frequency.

5.3.2 Sterilisation using a filter unit

In other studies that have investigated disinfection of insect eggs, it was recognised that not only the concentration of bleach used was important, but also the volume used and the thoroughness of the washing to remove traces of bleach following treatment was important (Hussa and Goodrich-Blair, 2012). Hypochlorite is unstable and it is possible that at low concentrations the concentration can decrease during incubation times, reducing the effectiveness of the sterilisation process. To use an increased volume of bleach, a 0.45µm filter top unit was used in which a continuous flow of fresh bleach could be maintained during the incubation time. Also, it allowed a large volume of distilled water to be passed

over the eggs to wash them following bleach treatment. Trials with this suggested that exposure of eggs to 0.6% bleach for 3 minutes produced bacteria-free eggs as assessed by incubating them on BHI agar (Figure 5.1). In addition, the hatching frequency was improved by this method (for example, see Figure 5.2).

A further validation of the sterilisation process was performed. Samples of 3 to 5 eggs were treated with 0.6% bleach and washed while retained on the tissue pieces onto which they were deposited by the adult moth. The tissue pieces were inoculated into 10 ml of sterile BHI broth using sterile disposable forceps and incubated at 30^{o}C. The OD_{600nm} of cultures was monitored but did not change over 5 days, indicative of the absence of bacteria.

To test whether the hatchling 1st instar larvae were free from bacteria, whole larvae (n=3 to 5/sample) were incubated in enrichment broths for 5 days, but no bacterial growth was observed.

5.3.3 The viability of large batches of eggs treated with 0.6% bleach.

The previous tests used low numbers of well separated eggs (n=5-15). For large scale experiments, larger batches of eggs will need to be used, that can be deposited in clumps. To test the efficacy of the sterilisation procedure on larger batches, the hatching frequency of batches of eggs treated with 0.6% bleach for three minutes was compared to control samples (n= up to 100 eggs/experiment/group). The viability % of bleached eggs was approximately two-fold less than that of controls (22.8% vs 51.6%) at day 4. The Holm-Sidak method with alpha of 0.05 was utilized to determine statistical significance between

both groups. A p value of 0.035342, SD= 0.813 was obtained which denoted statistical significance between both groups. At day 5 the HF of controls was 78.57% compared to 42.88 % for bleached eggs (n=100/group). A calculation of statistical significance with the Holm-Sidak method with an alpha of 0.05 demonstrated a calculated p value of 0.017931, SD= 3.663 which was demonstrated as being statistically significant (see Figure 5.2).

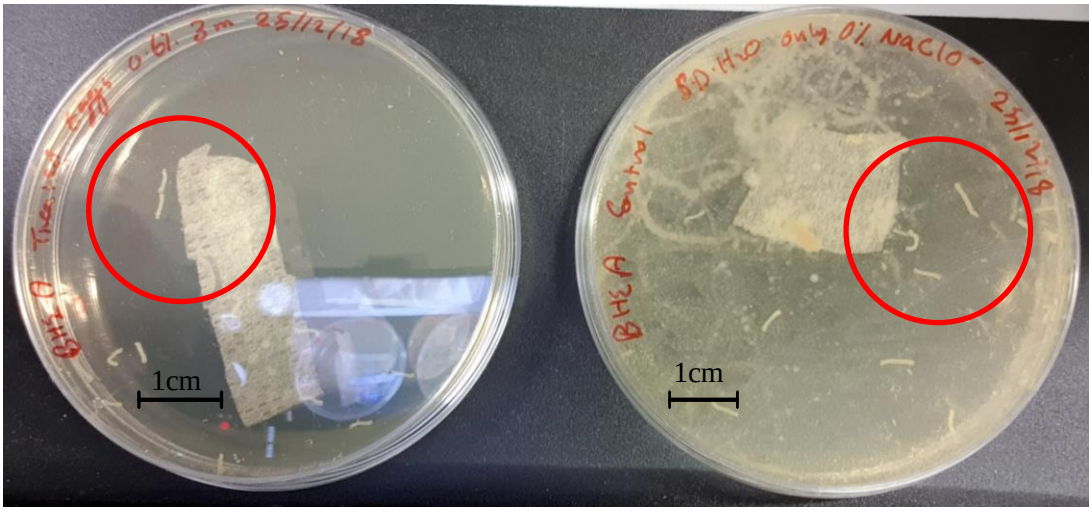


Figure 5.1. Photograph of BHI agar plates incubated at 26°C, 16h:8h dark: light period for 4 to 5 days until *M. sexta* eggs were hatched. On the left-hand side, (red circle)1cm hatchlings derived from bleached eggs (0.6% bleach, 3 m, n=90). On the right-hand side (red circle) are hatchlings 1cm derived from control eggs (sterile distilled water, 3m, n=93), (No contamination was observed for bleached eggs whereas extensive bacterial growth was observed with controls.

The combined findings of these studies resulted in a protocol for consistent elimination of egg-associated bacteria, which despite affecting the % of HF, enabled sufficient bacteria-free hatchlings to be recovered for further studies (> or = 50% of BF-1st instar larvae of the total number n= ~100/patch).

Figure 5.2: The hatching frequency of bleached and control eggs (n=100/group) was determined at days 4 and 5 (referred to as post and late hatching period). The HF of bleached eggs was two-fold less than that of the controls (p values 0.035342, SD= 0.813 at day 4 and P value 0.017931, SD= 3.663 at day 5). The reduction of the survival of the bleached eggs may be due to the damage caused by the bleach treatment and/or the absence of egg-surface bacteria.

Additional considerations were incorporated to create a consistent protocol for the generation of bacteria-free hatchlings:

1. Insect eggs are normally deposited by the adult moths onto a collection area (a clean white piece of tissue) that can be contaminated by moth faeces, and these contaminated areas were removed where possible.
2. Where possible, large clusters of eggs were avoided to allow the most thorough washing of each egg with bleach.
3. Eggs were collected and treated first thing in the morning to reduce the contamination from bacteria present in the moth enclosure.

4. During the 3 minutes of exposure to 0.6% bleach, the filter unit was gently agitated to improve washing.
5. The maximum number of eggs in a batch is approximately 100 (see Figure 5.3).

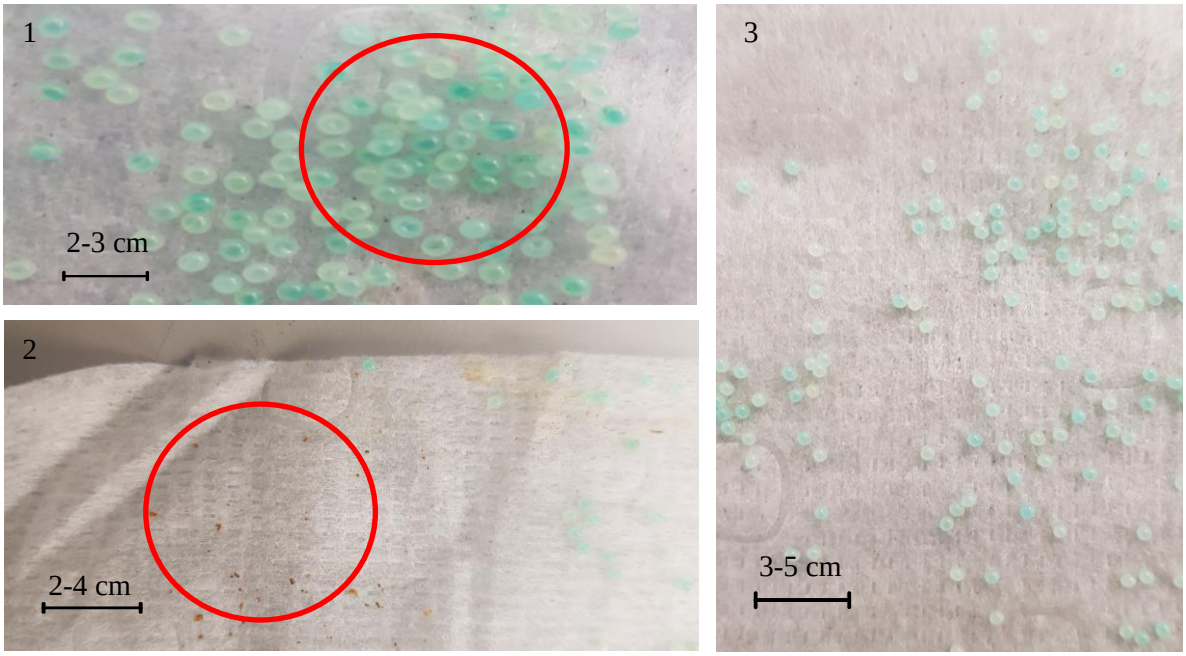


Figure 5.3: Image shows different areas of deposited *M. sexta* eggs on tissues that were collected from winged-cage moth. 1. Clusters of eggs (red circle) may prevent thorough washing. 2. An area of tissue contaminated by insect feacal material (red circle) which may increases the microbial load of the eggs sample. 3. A representive sample area with clean and separated eggs.

5.4 The growth of conventional rearing of Bath colony larvae

Under the laboratory condition at 26^{o}C, 16L:8D period, and 47% humidity, *Manduca sexta* larvae typically undergo five different larval stages prior eggs hatching stage (4 to 5 days). However, at late larval stages of growth and development (13, 15 and 17 days), the food consumptions by larvae rapidly increase as well as the weight gain. Thus, larvae become more distinguishable in terms of weight and size (see Figure 5.4). Moreover, the critical

body weight of the *M. sexta* larvae that occurs during moulting cycle before pupation has been documented and used as a proxy to determine when instar will moult to the next stage and the time between two successive moults is fixed for each different stage (i.e., 1st, 2nd, 3rd, 4th and 5th larval stage (Grunert *et al.*, 2015), see Figure 1.6 in chapter 1.

The artificial diet used to feed the laboratory *M. sexta* larvae is a well-defined formula and was designed specifically for breading the stock of this pest. This diet mainly consists of wheatgerm, water, agar and supplementations of antibiotics such as tetracycline, kanamycin or streptomycin sulphate to supress the gut microbiome of larvae and thus preventing the contamination of the colony. Additionally, casein, Linseed oil, cholesterol, Wesson's salt, vitamins such as Vanderaznt, riboflavin, biotin, folic acid and B12 are included in the food to support and maintain normal insect growth and development during the life cycle (Ahmad, Waldbauer and Friedman, 1989), see material and methods in chapter 2.

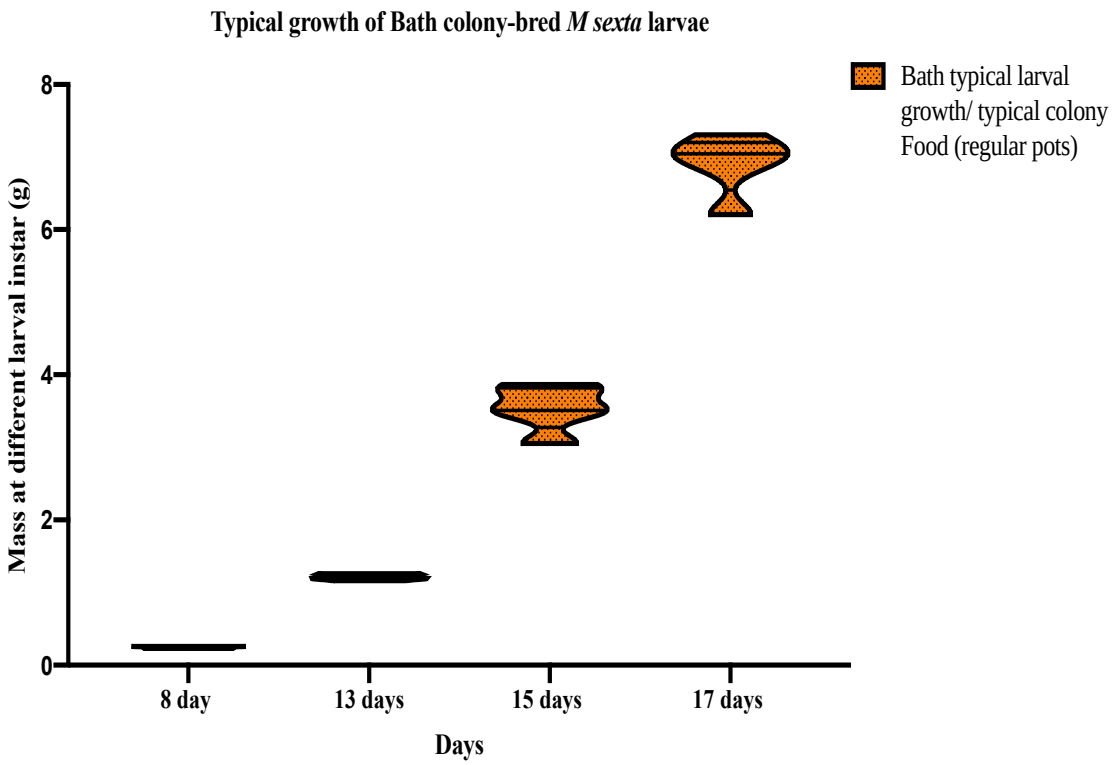


Figure 5.4: The growth of *M. sexta* larvae in the Bath colony is based on the data was determined during this study. The violin plots illustrate the frequency distribution of the median body mass of Bath colony *M. sexta* larvae (n=7) at different larval stages ranging from early 4th instar (8d), post 5th stage (13d) and late 5th instar (15-17d) respectively. The larvae achieve rapid weight gain during the late 5th instar stage.

5.5 The growth and development of bacteria-free *M. sexta* larvae.

The protocol described above enabled the hatching of larvae that are bacteria-free as measured by the inability to culture any bacteria from them. The effect of raising the larvae bacteria-free was investigated. Five groups of larvae (n=20/group) were reared:

G1: sterile (bacteria-free) hatchlings, raised on regular colony food (non-sterile, but tetracycline-supplemented) in standard clean pots: referred to as typical colony conditions.

G2: regular colony hatchlings fed on sterile food, in standard clean pots.

G3: regular colony hatchlings raised under typical colony conditions.

G4: sterile hatchlings raised on sterile food in barrier tubes.

G5: sterile hatchlings raised on sterile food, supplemented with an antibiotic cocktail, in barrier tubes.

The weight of each larvae was measured at the later stage of development, i.e., post-5th instar at days 13, 15 and 17. However, only larvae in groups 1 – 3 survived until the 5th instar stage of development (n=20/group), see Figure 5.5 and 5.6.

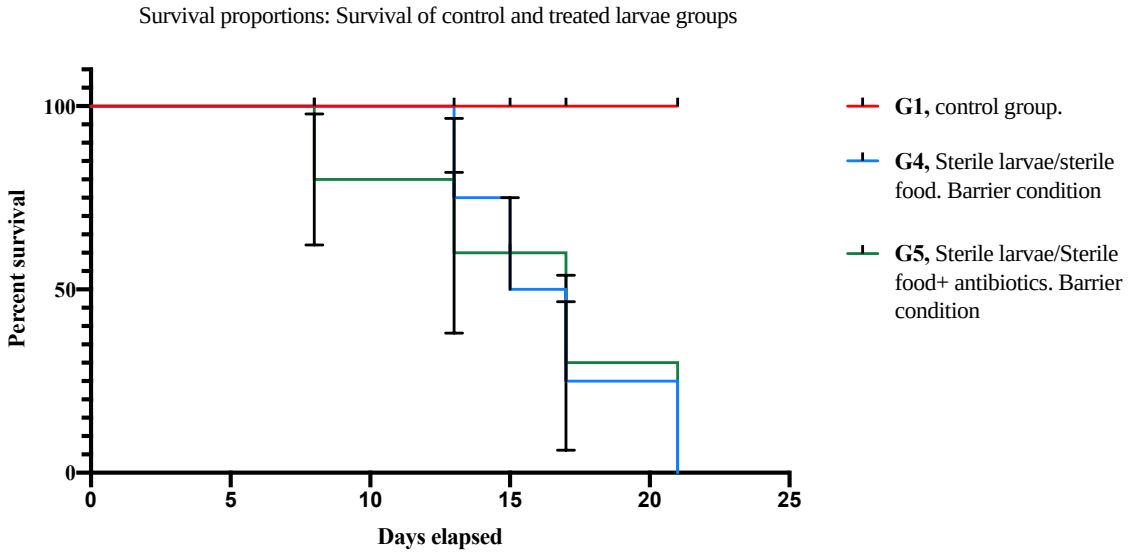


Figure 5.5: Survival of larvae in groups 4 and 5 compared to the control group 3 (n=20/group). A high mortality was observed in G4 and G5 where these larvae were started as bacteria-free fed without or with antibiotic sterile supplemented diet for all larval stages. Most of the larvae in Groups 4 and 5 died-off before reaching the 5th instar stage.

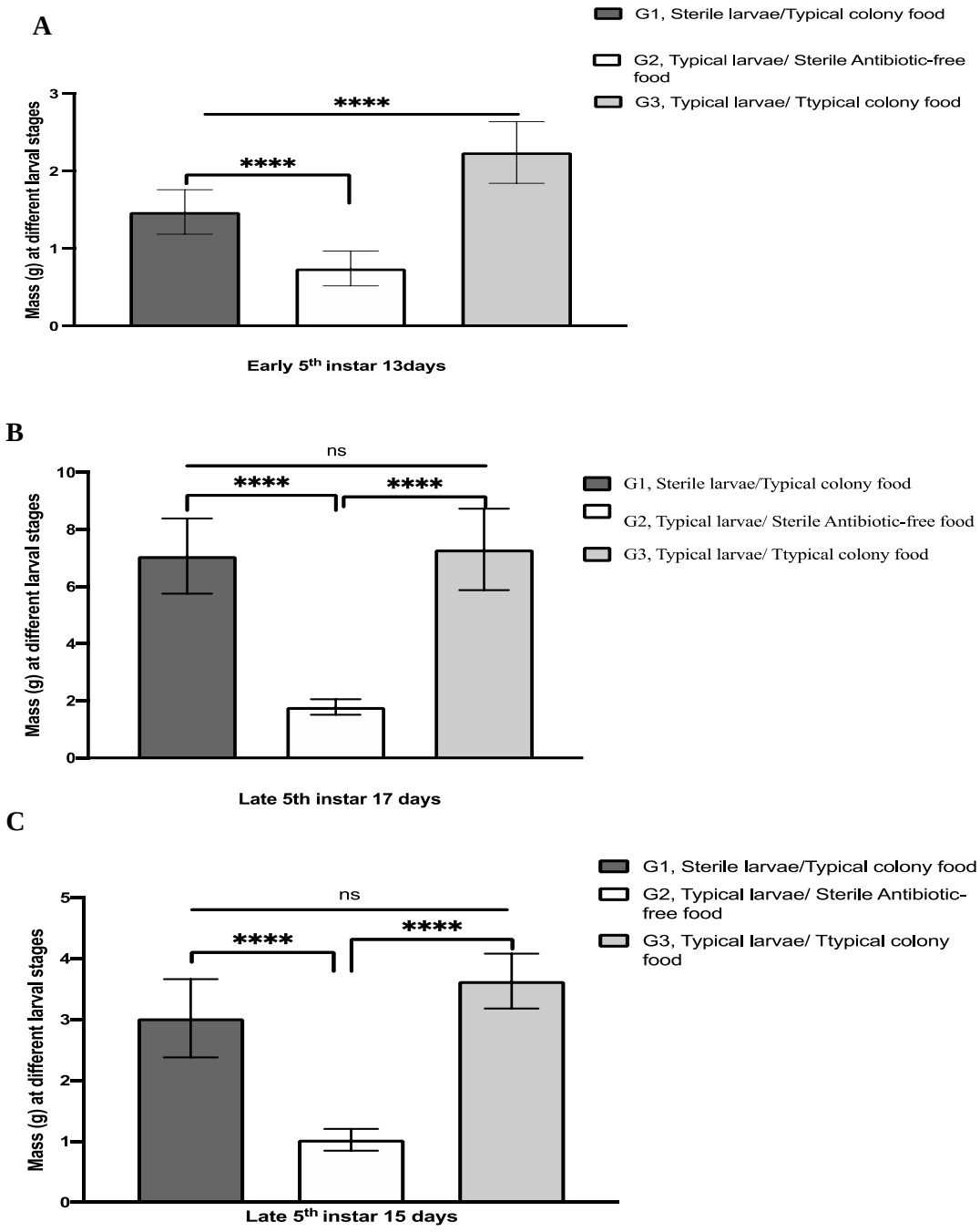


Figure 5.6: The average body weight (g) of larvae in each group (n=10/group) (**A**) at day 13, SD= 0.271, 0.213, 0.377 and ******** p<0.0001, (**B**) at day 15, SD= 0.608, 0.169, 0.426 and ******** p<0.0001, (**C**) at day 17, SD= 1.248, 0.257, 1.274 and ******** p<0.0001 for G1, G2 and G3 respectively using a two-way ANOVA test**.**

At day 13, the average weight of G1 was significantly lower than that of the control group G3 (n= 20/group, SD= 0.271, 0.213 and **** p<0.0001). However, at days 15 and 17 there was no difference between these groups (SD= 0.608, 0.426, 1.248, 1.274 and ns P>0.05 respectively). This suggests that during early development, the absence of bacteria in hatchlings slowed weight gain but these larvae appeared to reach the same final development weight as controls. Larvae in G2 (reared on sterile food), had significantly lower weights than either G1 or G3 at all time points **(**SD= 0.271, 0.213, 0.377 and **** p<0.0001. At day 15, SD= 0.608, 0.169, 0.426 and **** p<0.0001. At day 17, SD= 1.248, 0.257, 1.274 and **** p<0.0001).

5.6 Effect of removing gut bacteria during larvae growth.

To investigate the possible temporal effect of food-derived bacteria groups of larvae were grown in which larvae (n=40 in total) were reared under regular colony conditions until day 8, at which point they were randomly segregated into 4 different groups (n=10/group) for the remainder of their development:

G1: switched to sterile food and raised in barrier tubes.

G2: switched to sterile food supplemented with a cocktail of antibiotics and raised in sterile barrier tubes.

G3: switched to regular food supplemented with a cocktail of antibiotics raised in regular clean pots.

G4: control group, remained fed on regular food in regular clean pots.

The weight of larvae was measured. In this experiment later time points were used in the event of slower development of some groups. Thus, the larvae in each group (n=10/group)

were weighed on days 13 (SD= 0.084, 0.266, 0.0502, 0.233 and P>0.05 (ns)), day 17 (SD= 3.118, 0.609, 0.271 0.393 and **P<0.001) and day 21 (SD= 2.338, 3.991, 0.717, 0.678 and ***P<0.001) for G1, G2, G3 and G4 respectively, see Figure 5.7. The experiment was repeated, Figure 5.8 but with larvae measured at days 13 (SD= 0.059, 0.220, 0.048, 0.160 and P>0.05(ns)), day15 (SD= 0.406, 0.123, 0.5029, 0.7869 and ***P<0.0001) and day 17 (SD= 0.4426, 0.1115, 0.4504, 0.877 and ****P<0.0001) for G1, G2, G3 and G4 respectively to investigate the period when significant differences were observed in the first experiment.

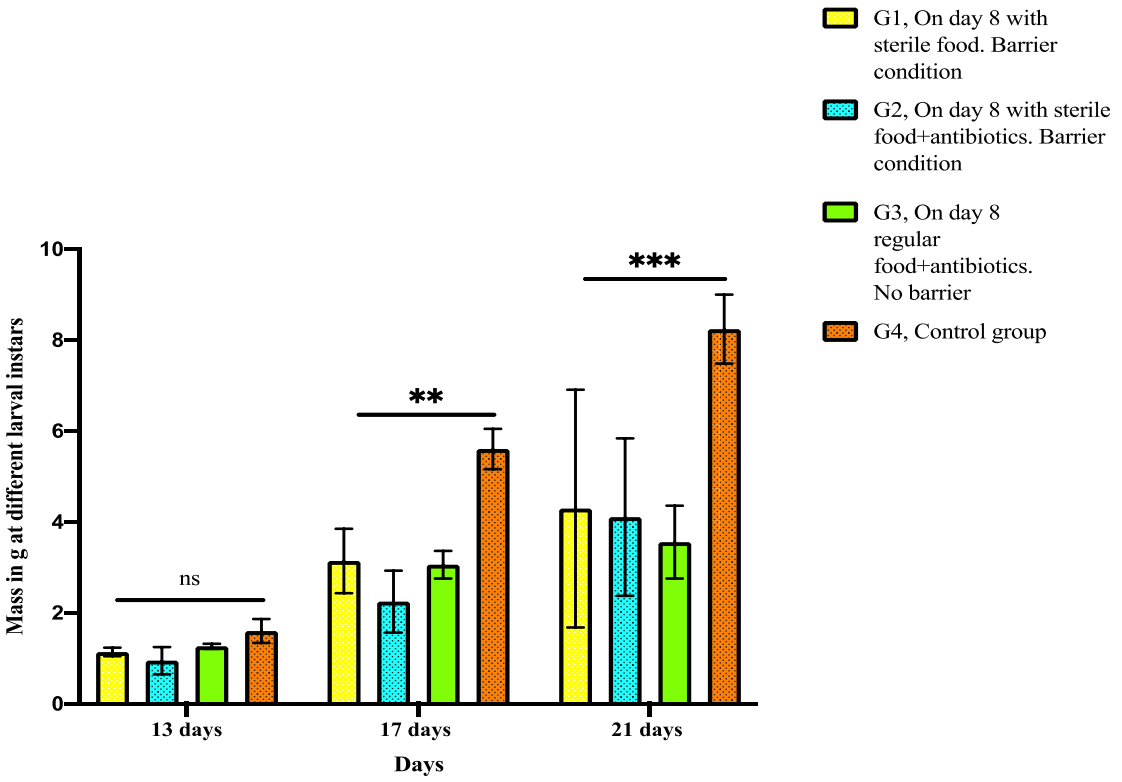


Figure 5.7: The average weight of post 5th instar larvae were grown under regular (typical) colony conditions until day 8 and then switched to different foods and/or barrier conditions. At day 13 (SD= 0.084, 0.266, 0.0502, 0.233 and P>0.045 (ns)), late 5th instar day 17 (SD= 3.118, 0.609, 0.271 0.393 and **P<0.001) and at day 21 (SD= 2.338, 3.991, 0.717, 0.678 and ***P<0.0001) for G1, G2, G3 and G4 respectively. Statistical significance is denoted by (***), while not significant is denoting with ns.

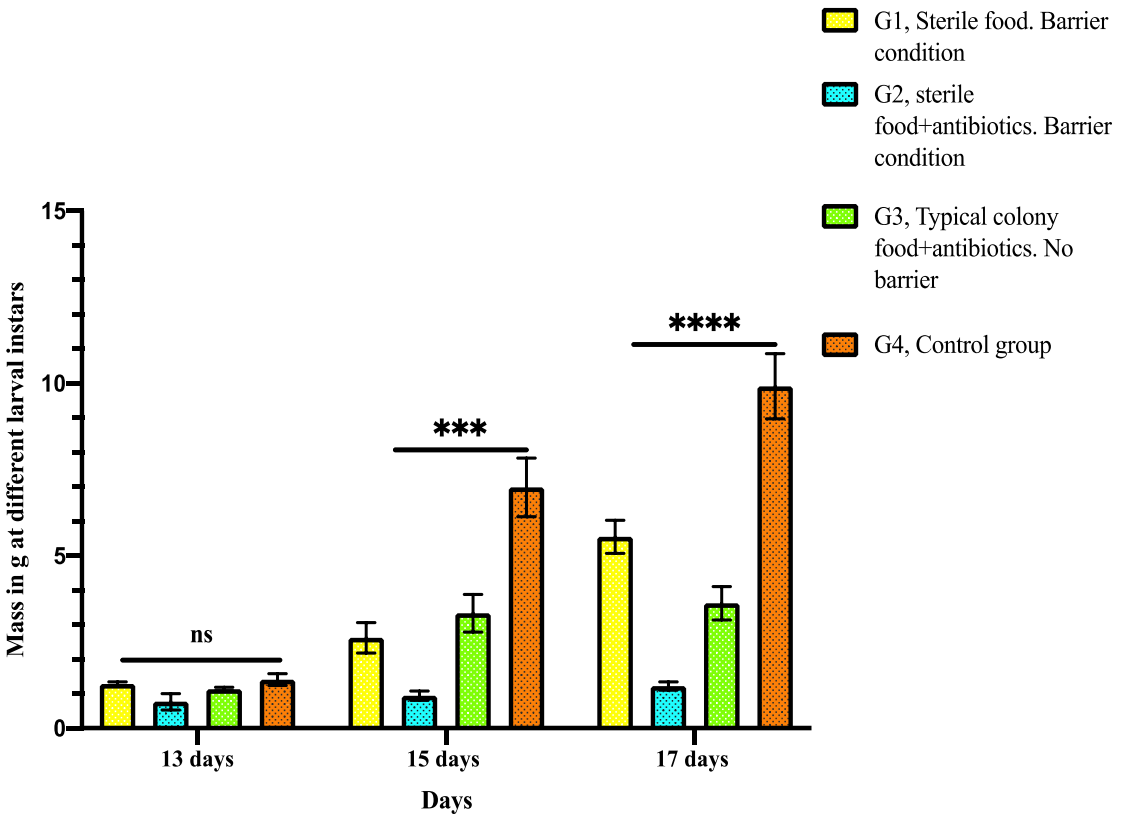


Figure 5.8: Repeat of the experiment depicted in Figure 5.7. The mean body weight of each larvae/group 5th instar stages of development. At day 13 (SD= 0.059, 0.220, 0.048, 0.160 and P>0.1085 (ns)), day15 (SD= 0.406, 0.123, 0.5029, 0.7869 and ***P<0.0001) and day 17 (SD= 0.4426, 0.1115, 0.4504, 0.877 and ****P<0.0001) for G1, G2, G3 and G4 respectively. Statistical significance is denoted by (****), whereas not significant is denoting with ns not-significant.

This experiment grew typical colony larvae until day 8 (n=40 in total, 10/group). These larvae were hatched from eggs contaminated with bacteria and raised on non-sterile food and exposed to other environmental bacteria. Before the 5th instar stage in which the most rapid period of growth happens, larvae were switched to different conditions, comprising sterile food and barrier conditions, sterile food supplemented with antibiotics and barrier conditions, and regular food supplemented with antibiotics in non-barrier conditions. Controls remained fed with regular food in non-barrier conditions. This experiment

focused on the role of food-derived bacteria (as opposed to egg-derived bacteria) in the development of 5th star larvae.

5.7 Effect of introducing gut bacteria during larval growth.

The previous experiment investigated the effect of removing gut bacteria at from day 8 on larval growth during the 5th instar stage. This experiment studied the effect of raising bacteria-free larvae for the first 8 days of growth and then introducing gut bacteria, to investigate if the absence of bacteria during early growth had lasting effects.

Bacteria-free hatchlings (n=10/group) were reared on sterile food, with or without antibiotics, under barrier conditions and then switched to typical colony food/rearing conditions prior to 4th instar larval stage (day 8). Or typical larvae were hatched but fed with typical food supplemented with antibiotics under non-barrier conditions until day 8 after which they were fed with typical colony food:

Table 5.2: The weights of the larvae (n=10/group) were measured at day 8 and during the 5th instar stage and the experiment was repeated but with an extended time for the second experiment to account for slow growth of some larvae, figures 5.9 – 5.11.

Group	Conditions up to day 8	Conditions after day 8
1	Typical larvae, typical colony food, non-barrier conditions	Typical colony food, non-barrier
2	Sterile larvae, sterile food. Barrier conditions	Sterile larvae, sterile food. Barrier conditions.
3	Sterile larvae, sterile food. Barrier conditions	Typical colony food, non-barrier
4	Sterile larvae, sterile food plus antibiotics. Barrier conditions	Sterile larvae, sterile food plus antibiotics. Barrier conditions
5	Sterile larvae, sterile food plus antibiotics. Barrier conditions	Typical colony food, non-barrier
6	Typical larvae, typical colony food plus antibiotics, non-barrier conditions	Typical larvae, typical colony food plus antibiotics, non-barrier conditions
7	Typical larvae, typical colony food plus antibiotics, non-barrier conditions	Typical colony food, non-barrier

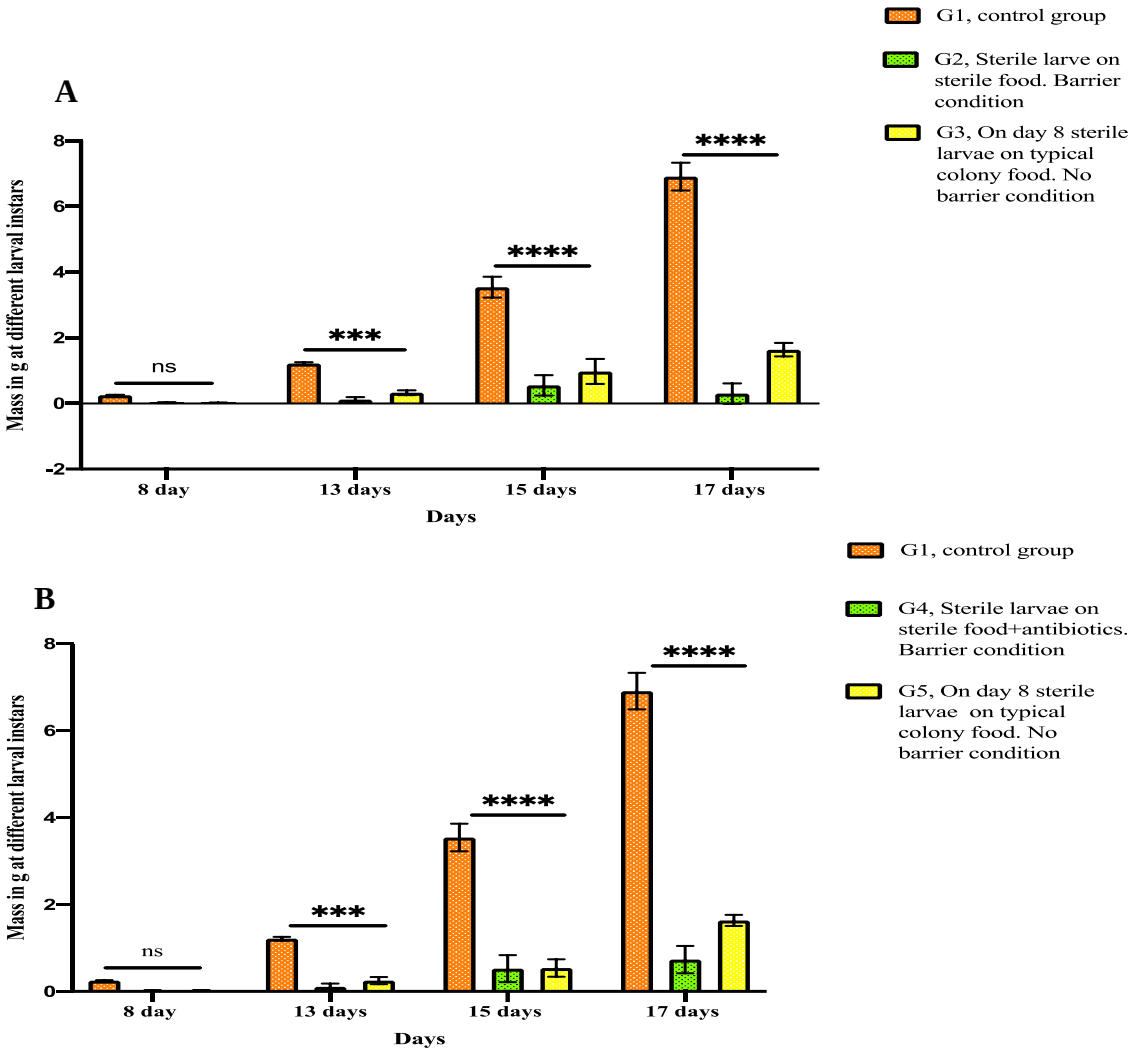


Figure 5.9: Comparison of the weights of larvae during 5th instar stage (n=10/group). (A) 1st experiment, at day 8 (SD= 0.012, 0.1011, 0.005 and P> 0.05 (ns), at day 13 (SD= 0.036, 0.067, 0.065 and *** p<0.001, at day 15 (SD= 0.283, 0.2778, 0.3409 and ****p<0.0001, at day 17 (SD= 0.375, 0.2765, 0.1828 and **** p< 0.0001 for G1, G2 and G3 respectively. While (B) at day 8 (SD= 0.0122, 0.0068, 0.0049 and P> 0.05, ns: not-significant, at day 13 (SD= 0.067, 0.068, 0.074 and ****p<0.0001, at day 15 (SD= 0.283, 0.276, 0.178 and ****p<0.0001, at 17 (SD= 0.375, 0.280, 0113 and ****p<0.0001 for G1, G4 and G5 respectively. Groups are explained in the table 5.2.

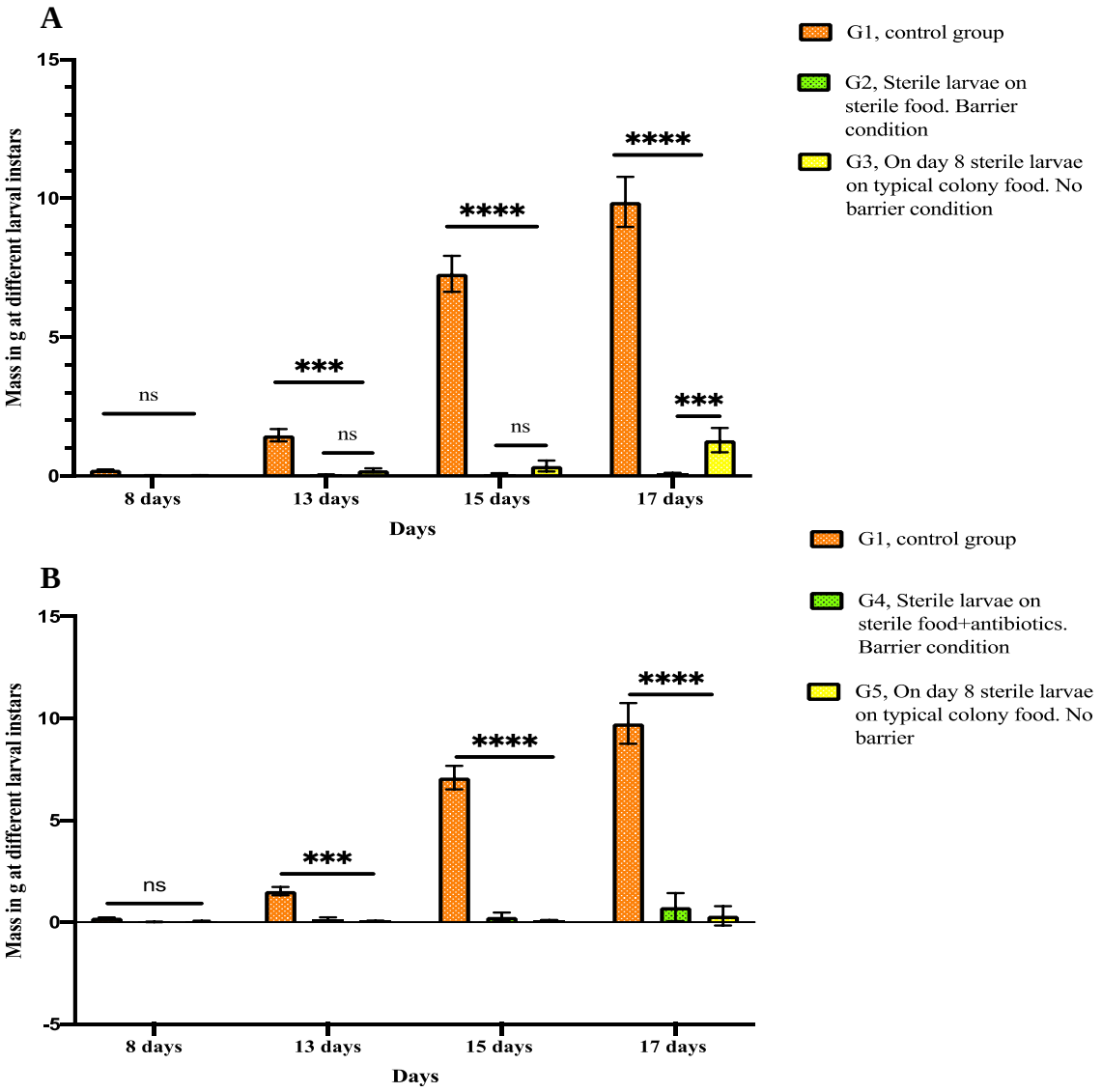


Figure 5.10: Repeat experiment, comparison of the weights of larvae during 5th instar stage (n=10/group). (A) at day 8 (SD= 0.0122, 0.10058, 0.0049 and P>0.05, ns: not-significant, at day 13 (SD= 0.4856, 0.0687, 0.0741 and ***: p<0.01, at day 15 (SD= 0.9347, 0.276, 0.1788 and **** p< 0.0001, at day 17 (SD= 0.898, 0.280, 0.1136 and **** p< 0.0001 for G1, G2 and G3 respectively. While (B) at day 8 (SD= 0.0122, 0.0055, 0.0116, P>0.05, ns: not-significant, at day 13 (SD= 0.4856, 0.0687, 0.0671 and **** p< 0.0001, at day 15 (SD= 0.9347, 0.276, 0.2778 and **** p< 0.0001, at day 17 (SD= 0.898, 0.280, 0.2765 and **** p< 0.0001 for G1, G4 and G5 respectively. Groups are explained in the table 5.2.

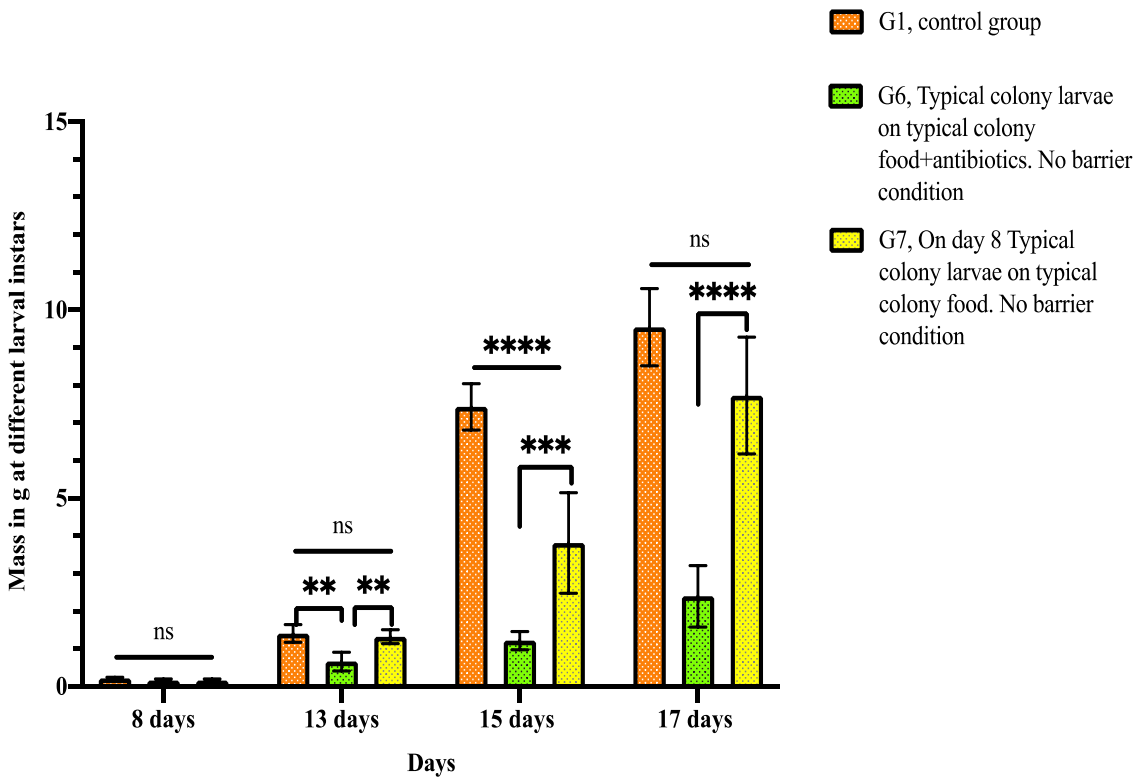


Figure 5.11: Comparison of the weights of larvae during 5th instar stage (n=10/group). First experiment only. At day 8 (SD= 0.0122, 0.0379, 0.0379 and ns: not-significant, at day 13 (SD= 0.4856, 0.23038, 0.1744 and ***: $p<0.001$, at day 15 (SD= 0.9347, 0.2241, 1.2367, and **** $p<0.0001$, at day 17 (SD= 0.898, 0.7548, 1.4362 and **** $p<0.0001$ for G1, G6 and G7 respectively. Groups are explained in the table 5.2.

In each experiment the sterility of larvae that remained under sterile conditions was verified by placing randomly selected larvae from each of these groups (n=3-5/medium) into enrichment broths and incubating at 30^{o}C for 5 days. In each case, no bacterial growth was observed, confirming that these groups of larvae had remained bacteria-free, except for Group 6. In the second experiment, contamination was observed for both groups 6 and 7.

This was traced to the antibiotics used to supplement the food being ineffective. Thus, only one set of data is shown for Groups 6 and 7.

The experiment included three different sterile larvae groups (n=10/group): sterile larvae fed on sterile food with or without antibiotics, and typical larvae fed antibiotic supplemented food that will be exposed to environmental bacteria but for which antibiotics in the food will eliminate a big majority of them.

5.8 Chapter summary and discussion

Bacterial symbionts can have a great impact on the growth and development of their respective hosts. The composition of the gut microbiome is varied across the entire animal kingdom with more than 1000 bacterial phenotypes identified in humans to a few tens in lepidopterans (Voirol *et al*., 2018). Gut bacteria play an important role in the digestion of nutrients and the provision of digestive enzymes that are necessary for vitamin synthesis and the increase of nutrient uptake in the gut (Engel and Moran, 2013). The first germ-free animal models that were developed were germ-free mice models. These models are bred in incubators and must be kept under such conditions to prevent the colonization of the gut by external microbial sources. An alternative method that has been derived for the generation of germ-free mice models is through the use of broad-spectrum antibiotics which are used to eliminating the gut microbiota of the mice. Germ-free mice show an impaired early development of the immune system while antibiotic treated mice have added advantage of allowing the study of immune-related functions of the host gut microbiome after development (Kennedy, King and Baldridge, 2018). Insight gained from mice model on the role of the gut microbiome on the host e.g., development, immune response, metabolism and the maintenance of normal host physiology. However, the complexity of the gut microbiome compositions of these higher vertebrate models adds further challenges in such research field. In particular case of the cause and effect of specific bacterial species that can attribute to the e.g., obesity in human (causality). It has been proven that obesity symptoms often associated with the change of structure and functions of human intestinal microbiome (e.g., relative ratio of main phyla of *Bacteroides* and *Firmicutes*). Thus, there has been a need for an alternative invertebrate model that host

relatively simpler gut microbiome community (Kostic, Howitt and Garrett, 2013). One such models that was earlier established was the dragonfly *Libellula Pulchella*, which was observed to display similarities in metabolic syndrome and obesity as mice when infected with an intestinal protozoan. Infection of *Libellula Pulchella* with a common insect gut protozoan (Apicomplexa Eugregarinorda) aimed at determining the impact of this parasite on the overall performance and physiology of the dragonfly. The lack of lipids digestion in these dragon flies was associated with the increase number of intestinal protozoans and resulted in reduced fat metabolism and increased weight gain. While it was additionally observed the induction of pro-inflammatory cytokines such as P38, MAPK and the C-Jun N-terminal kinase pathways as what was observed in germ-free mice. These results clearly demonstrate that changes in structure and function of the host gut microbiome cause changes in host homeostasis and metabolism not only in mammals but in insect models as well. Thus, it has been concluded that the use of insect model system with relatively less complex gut microbiome would address the key research questions concerning the role of gut microbiome in higher vertebrate (Schilder and Marden, 2007).

5.8.1 Effect of sterilizing the eggs of *Manduca sexta* with bleach solution

The protocol to establish germ-free insects and to study the host gut microbiome often involves the preparation of a sterile incubator that will house respective sterile larvae, collection, separation of embryos, and egg bleaching. Egg bleaching is the first and important step in this process, in order to prevent the acquisition of bacteria located on the egg surface from colonizing the gut of the newly hatched larvae. It has been suggested that the transmission rout of insect gut bacteria can be horizontally from e.g., food, between

individuals or maternally/vertically via eggshell layer (Brinkmann, Martens and Tebbe, 2008; Voirol et al., 2018). Despite the use of bleach to reduce the presence of bacteria on the egg surfaces, traces of bacteria were still observed when the eggs were washed with less concentrations of bleach and different exposure times, see Table 5.1 (Brundage, Crippen and Tomberlin, 2016). The use of relatively higher concentration of bleach for 3 minutes exposure (>0.55%) resulting in tentatively sterile eggs as no microbial growth was seen on agar plate (up to n=100/group). Even though, the HF% of the treated eggs was markedly lower compared to the HF% of control eggs (sterile distilled water). While, using enrichment broth cultures to further confirm the sterility of the resulting dechlorinated eggs was indicated to the presence of yet low number of bacteria was remained on the egg-shell layers. Indeed, using 0.45µm top filter unit as it described in the study conducted by Hussa and Goodrich-Blair. (2012) was further improved the thoroughness of washing step (dichlorination) and relatively increased % of eggs viability. Regardless of using this method that was allowed a large-scale experiment to conduct (~100 eggs/patch). Yet the hatching percentage of bleached eggs was still tow fold less than that of the control eggs (22.8% vs 51.6% at day 4, p value of 0.035342, SD= 0.813 and 42.88 % vs 78.57% at day 5, p value 0.017931, SD= 3.663), see Figure 5.2. This was most likely due to the fact that the high concentration of the bleach solution can damage the eggshell thus leading to the death of the developing larvae. Additionally, bleach should not be directly used to sterilize freshly laid eggs, given the fact that the eggshell is still soft, and this can lead the entry of bleach directly into the developing embryo (Brundage, Crippen and Tomberlin, 2016). Other processes to ensure that the eggs are sterile before hatching are dichlorination, which is a process whereby, the eggshell layer is manually removed, and this process is typically

carried out under sterile conditions in a laminar hood. The bleached embryos are then transferred into sterile incubators where they are housed and fed on food infused with antibiotics (Sabat et al., 2015). Thus, the bleach concentration and exposure time parameters should optimise during sterilisation process in which a reasonable viability (%) of bacteria-free eggs can be determined (Brundage, Crippen and Tomberlin, 2016; Hussa and Goodrich-Blair, 2012). Alternatively, in a study by Salem et al. (2013) determined that the symbiotic relationship between egg surface bacteria and the hatching frequency of the eggs of firebugs and cotton strainers. It was observed that elimination of symbionts by egg-surface sterilization resulted in a higher mortality, reduced hatching frequency and reduced growth rates of the larvae compared to control samples wherein the eggs were washed with sterilized distilled water. It was concluded that the microbial community might play an important role for host nutrition and as such, elimination of this microbial community might result in poor nutrient availability of the unhatched eggs (Salem *et al*., 2013). It is hypothesized that the resident gut microbiome of the larvae is obtained from egg-surface host microbial communities which can interact with the larvae when still contained in the egg surface. These microbial communities establish themselves in the developing gut of the larvae before and after the hatching process and thus facilitate nutrition due to their ability to support the breakdown of essential nutrients which might be required for the development of the naïve egg into the larval stages.

5.8.2 Assessing the growth and development of BF-*M. sexta* larvae.

These results of this experiments demonstrated a significant effect of raising larvae from bacteria-free eggs on sterile food. It has previously been reported that *M. sexta* contains a low-density gut microbiome and that the gut was most likely colonized by microorganisms present in their conventional food that was fed to the larvae and not as a result of resident gut microbiota present in the larvae (Brinkmann, Martens and Tebbe, 2008; Tang et al., 2012; Hammer et al., 2017). These bacteria were absent from G2. The low body weight of these larvae suggests that these bacteria might be essential for the conversion of macromolecules contained in the food into simpler components that might be easy to digest and absorb in the gut of the larvae and their absence significantly affects the nutrition of the larvae.

The initial low weight of G1 larvae (sterile hatchling reared on regular food) might suggest that during early growth egg-derived bacteria contribute to larval growth and development. During this time, the larvae are small and consume relatively little food compared to late-stage larvae, thus egg-derived bacteria might be important during this stage. During the later stages of growth, food consumption increases rapidly and thus the levels of food-derived bacteria will also increase rapidly. G1 larvae reached the same final weight as control larvae suggesting that during later development, food-derived bacteria play a major role in growth.

Groups 1, 2 and 3 had some exposure to bacteria. In group 1, the bacteria-free hatchlings were fed regular colony food that was not sterile. Group 2 comprised non-sterile larvae fed on sterile food while the control group 3 comprised non-sterile larvae fed regular colony food. Interestingly, groups 4 and 5 were designed to be bacteria-free throughout comprising

bacteria-free hatchlings fed on sterile food, either with or without a cocktail of antibiotics. These larvae displayed increased mortality compared to those exposed to bacteria, with very few even reaching the 5th instar stage. This suggests a role for microbiome bacteria in the development and growth of the Bath colony *M. sexta* larvae.

The results from these studies clearly demonstrate that bacteria present in the typical colony food are capable of colonizing the gut of the larvae of *Manduca sexta*, whether these larvae are grown under sterile or non-sterile conditions. These bacteria might thus play an important role in the host metabolism by synthesizing digestive enzymes that enable the production of vitamins and other nutrients which are essential for the growth and development of the bacteria. Several studies have attempted to elucidate the role of the gut microbiome in terms of host metabolism, growth and development in insects. For example, in aphids, *Buchnera* bacteria were observed to play a role with the provision of essential amino and vitamins (Hansen and Moran, 2013). Gut microbiomes might also provide a metabolic benefit to their hosts by synthesizing digestive enzymes and vitamins which might lead to an increase in nutrient uptake and a direct effect on the weight of the host, which might be what was observed in the *Manduca sex*ta larvae in these studies (Engel and Moran, 2013). In the sterile fed exclusively with sterile food supplemented with (G5) or without being antibiotics treated (G4), these larvae failed to thrive, and lose weight gain as shown in their counterparts that were fed with sterile food in comparison to the G1 larvae (regular colony food). Kaufman, Klug and Merritt, (1989) conducted a study wherein the germ-free larvae of *Acheta domesticus* was reared under different diet conditions. It was observed that non-germ-free crickets reared under normal conditions (i.e., colony food, pots, etc) showed the highest growth rate than that of germ-free larvae (i.e., barrier, sterile

food). In terms of the food utilization parameters, conventional larvae digested a greater proportion of diet which was readily converted into essential carbohydrates, in comparison to germ-free larvae (Kaufman, Klug and Merritt, 1989).

5.8.3 Effect of depleting the gut microbiome during the growth of the larvae.

As expected, the control larvae displayed normal growth, with rapid weight gain and size increase during the 5th instar stage. At day 13 the other groups had very slightly lower weight than controls, but the difference was not significant. However, at day 17 all groups had significantly reduced weight gain compared to control larvae. This deficit remained through till day 21. The three groups were designed to prevent the introduction of food-derived bacteria during this period and groups 2 and 3 were also fed on antibiotic supplemented food that would eliminate bacteria from the larvae guts. We hypothesize that the antibiotics cocktail might be successfully suppressed the gut microbiome bacterial load of the guts of these larvae and thus the weight loss occurred due to the absence of a resident gut and the lack of food-borne bacteria contributed to the significant weight loss observed in these groups G2 and G3. These larvae might not contain either a resident gut microbiome or food-borne bacteria to digest the nutrients obtained from the typical colony food. As such, these larvae might have a lower nutrient availability due to the lack of gut bacteria compared to the control group larvae (G4) that were rapidly increased weight gain particularly at late 5th instar. While G1 larvae that were reared only with sterile food still but without antibiotic might have earlier an intact resident gut microbiome which was capable of digesting the nutrients from the sterile food. However, given the fact that the weight of these larvae was reduced compared to the control group (G4), we hypothesize

that the resident gut microbiome and food-borne are both essential for the digestion of nutrients from food sources and the absence of either bacterial sources might lead to a reduction in nutrient availability and hence a decrease in weight gain of the larvae (Habineza *et al.*, 2019). These results strongly implicate gut bacteria in the growth of larvae during the 5th instar stage. This is the period of fastest growth of *M. sexta* larvae, and thus presumably digestion of food and absorption of nutrients is critical. It has been reported that the development of insect gut microbiome not only influences by diet, habitat, and phylogeny but it might be by the developmental stages of the host (Yun *et al.*, 2014; Voirol *et al*., 2018).

5.8.4 Effect of introducing gut bacteria during the growth of the larvae

For Groups 2 and 4, comprising sterile larvae fed on sterile food, the same poor growth was observed as in previous experiments. The weight of the larvae was significantly lower than control larvae at all 5th instar stages, although they did increase in weight during this time. For both groups (G3 and G5), switching the larvae to typical colony food and non-barrier conditions from day 8 resulted in increased weight gain compared to controls that were not switched suggesting that the introduction of bacteria post day 8 facilitated larval growth during 5th instar. However, the weights of these larvae remained significantly lower than control larvae (G1), raised under typical colony conditions throughout, revealing that the absence of bacteria during the first 8 days of growth and development has effects that last throughout 5th instar that are not alleviated by introducing bacteria from day 8 onwards.

An interesting result was observed for larvae that were raised under typical conditions except for supplementation of the typical colony food with a cocktail of antibiotics (G6

and G7). These larvae were not significantly different from control larvae in terms of weight at day 13, whereas the sterile larvae groups (G2-5) were significantly lighter than controls by this stage. At day 15 however, G6 and G7 larvae were significantly lighter than controls but with the switched group (G7) significantly heavier than the group kept on antibiotic-supplemented food (G6). By day 17, the Group 7 larvae were not significantly different in weight to the controls, whereas G6 showed continued poor growth. The difference between these larvae and those in Groups 2-5 is that they were exposed to bacteria during the first 8 days of growth, even if the antibiotics present in the food prevented carriage of viable bacteria in their guts. This appears to have primed these larvae to achieve normal weight gain on the introduction of viable bacteria after switching to antibiotic free food. Priming alone did not stimulate normal weight gain, as this was observed only after the removal of antibiotics from the food at day 8. Caterpillars deprived of bacteria either by maintaining sterile conditions or by supplementation of food with a cocktail of antibiotics demonstrated significantly reduced growth compared to controls. In a previous study by Cooper et al. (2017) it was demonstrated that the priming of *Manduca sexta* caterpillars with a non-pathogenic strain of *Escherichia coli* led to a long-term response to virulent insect pathogens which can be attributed to the strong antimicrobial effect of the insect haemolymph (Cooper and Eleftherianos, 2017). Despite the fact that sterile larvae might contain an underdeveloped immune system and decrease e.g., phenoloxidase and haemolymphs. Thus, these larvae might become more susceptible to insect pathogen and might massively die-off when challenged with a bacterial pathogen, demonstrating once more the importance of a resident gut microbiome not only in terms of

nutrient availability but the development of the immune system as well (McMillan and Adamo, 2020).

We hypothesize that groups of larvae grown firstly under sterile conditions lack a resident gut microbiome which might have led to an impaired development of the gut tissue and despite the late re-introduction of typical colony food derived bacteria (day 8), larvae failed to thrive and gained weight due to the inability of the gut to absorb nutrients derived from food sources metabolized by bacteria present in the food sources. Studies from other germ-free pest such as red palm weevil, *Rhynchophorus ferrugineus* (Olivier) has demonstrated that these germ-free larvae were fed with sterile food with or without a cocktail of antibiotics and maintained under sterile condition possessed a remarkable weight-loss compared to the colony control larvae group (regular food), thus demonstrating that the absence of a resident gut might impair nutrient availability in the haemolymph (Habineza *et al.*, 2019). Upon the reintroduction of the gut microbiome into the germ-free insects, the levels of nutrition were observed to be enhanced with increased survival rates. Interestingly, germ-free larvae that were associated with *Lactococcus lactis* were possessed similar level of proteins as that were observed with regular larvae reared on non-sterile food. Whereas those of gnotobiotic larvae that were fed on food contained *Enterobacter cloacae* demonstrated the same levels of lipid and carbohydrates as well as regular larvae were fed on colony food. These results from this study clearly demonstrate that the gut microbiome of pests plays an important role in the digestion of complex food and the regulation of the metabolism, growth and development (Habineza *et al.*, 2019). These experiments reveal a role for viable bacteria in promoting growth and weight gain of the

larvae, and for exposure to bacteria during early growth in priming larvae for growth and later in development.

Together these studies demonstrate that normal growth of Bath colony *M. sexta* caterpillars is dependent on the presence of live bacteria in their guts, particularly during 5th instar, the period of highest food consumption and growth.

Chapter 6. Conclusion and Discussion

The recent explosion of interest in the role of microbiomes in the health and disease of complex organisms has highlighted that simple, manipulatable models for microbiome research are needed. For studies of the human microbiome, the mouse model is often used as a surrogate model. However, alternative methods have been developed to generate germ-free mice models by depleting their resident gut microbiome using a cocktail of antibiotics. This method might permit the conservation of adult morphological features that might be essential to study the effect on depleting the gut microbiome on the host homeostasis (Kennedy, King and Baldridge, 2018). While the complexity of the mouse microbiome makes it a good model, but this complexity can also make it a difficult model in which to study cause and effect relationships between microbiome components and specific traits (causality). Also, the cost of using mice and ethical considerations can limit the use and access of the mouse model.

The University of Bath houses the only colony of *Manduca sexta* in the UK and the EU. This colony has been maintained under isolation for decades, producing a genetically pure colony. *M. sexta* is a model organism for studies of development and immunity. It is relatively cheap to maintain. It has relatively quick life cycle and it is easy to raise large numbers of larvae. Their large size makes them easy to handle, and they are genetically manipulatable. Previous studies had suggested that the microbiome of *M. sexta* was simpler than mammals. These features suggested *M. sexta* as a potential model organism for microbiome research. The aim of this thesis was to investigate the use of *M. sexta* for

microbiome studies. Prior to my study the role of the gut microbiome of *M. sexta* in host health and physiology was unclear.

For studies pertaining to the role of the gut microbiome in insect development and immunity, germ-free *Drosophila melanogaster* has been utilized to study the role of the gut microbiome not only in immunity but in terms of the growth and development of the insect. The majority of the bacterial species identified from the gut of *Drosophila melanogaster* were obtained from food sources (Corby-Harris *et al*., 2007). The dragonfly *Libellula Pulchella* has also been utilized to study the effect of altering the gut microbiome of the host through the introduction of an intestinal protozoan and its effect on the metabolism of the insect. It was observed that the introduction of the intestinal protozoan *Apicomplexa Eugregarinoda* in the gut of the dragon fly caused decreases in fat metabolism and increased obesity in these invertebrates thus demonstrating that alteration of the gut microbiome has an effect on host physiology (Schilder and Marden, 2007). In terms of the comparison of the gut microbiome of mice and insects, the gut microbiome of insects tends to be less complex than higher vertebrates and is not only constituted of bacteria species but composed of other microbes such as archaea, fungi, protozoa and viruses that are essential for the insect's fitness. Many examples of such microbes that are providing host with nutrients, bacteria such as *Buchnera aphidicola* in aphid that aid in metabolising and providing the amino acid tryptophan, which is not present in phloem, and the yest which is present in unripe olives and is readily ingested by the larvae of *Bactrocera oleae* upon hatching on unripe olives (Gurung and Falcao Salles, 2019). In mice on the other hand, the healthy gut microbiome was observed to consist of 37 bacteria genera such as *Bifidobacterium, Lactobacillus, Clostridium, Escherichia, Clostridium* XIVa,

Faecalibacterium, Ruminococcus, Bacteroides, Alistipes, Parabacteroides, Roseburia, Lachnospiraceae, Sporobacter, Dorea, Clostridium, Eubacterium, Collinsella, Coprococcus, Subdoligranulum, Streptococcus, Holdermania, Butyrivibrio, Anaerotruncus, Enterococcus, Blautia among other bacteria genera (Wang *et al.*, 2019).

Several *in vitro* culture conditions were applied to culture and identify gut microbiota isolated from *Manduca sexta* larvae that were reared under different diet systems (with or without antibiotic) in chapter 3. Despite the fact that diverse bacterial colonies were isolated from the gut of laboratory reared *M. sexta* larvae were predominant spore-forming bacteria belonging to the genera *Bacillus, Lysinibacillus, Enterococcus, Viridibacillus*, other types of bacteria include lactic acid bacteria, *Staphylococci*, Gram-negative *Pseudomonas* and a novel species of bacteria was also identified (*Oceanobacillus. massiliensis*). While the culture-free and enrichment broth culture-based methods were utilized whereby a wider gut microbiome profile was anticipated to be seen, yet the new species (*Oceanobacillus. massiliensis)* was not among those isolates isolated and identified in chapter 4. While several difficulties were encountered when carrying out the *in vitro* enrichment broth culturing and culture-free methods in order to identify and characterize the gut microbiome in *Manduca sexta* was due to low cell samples of larval gut content. Interestingly, despite that similarities and differences were seen among gut bacteria of *M. sexta* larvae, however the *Firmicutes* bacteria were still the most predominant phylum in all 5th instar larval gut across all samples. In chapter 5, we observed that 1st instar bacteria-free *M. sexta* larvae fed with typical colony food and reared in typical colony conditions showed the greatest weight gain as that of 1st instar typical colony larvae fed with typical colony food (control group). These results clearly demonstrated that the food-borne

bacteria colonies were presumably acquired as early as 1st stage larvae upon ingestion of the typical colony food might be essential in nutrient availability and maintaining the gut metabolism of the larvae and this might explain the weight gain acquired in theses larvae fed on typical food. However, not all the bacteria present on the egg surface might have been removed particularly when using the conventional colony disinfection method. This was due to the fact that viable bacteria might still remain on the egg surface and the larvae might be exposed to bacteria present in the growth chamber and as such, the bacteria species that were identified from the gut of the larvae might be emerged as both food-borne bacteria derived from the typical colony food and bacteria obtained from the environment where the adult moths are housed. When carrying out a comparison of the gut microbiome of the Lepidoptera species including *Manduca sexta* demonstrates differences with other invertebrate species. Several studies reported that the gut microbiomes of Lepidoptera in general were simpler than other animals and comprised mainly bacteria obtained from their food. For example, the gut of the moth *Heliothis virescens* was observed to contain bacteria species obtained exclusively from food sources or from the host environment (Staudacher *et al*., 2016). The gut of higher termites consists of bacteria and archaea that might play an important role in nutrient availability and resistance to pathogens (Brune and Dietrich, 2015). The physiology of the larval gut was described as very harsh for bacterial survival, containing numerous antibacterial components derived from host and consumed plant material (Voirol et al., 2018). The pH of the larval gut is high, in some reports as high as pH10, thought to be important for degrading plant material in their food (Chen *et al*., 2016). The simple structure of the larval gut and fast transit time led to suggestions that bacteria were transient passengers through the gut rather than adapted residents as observed in many

other animals (Hammer et al., 2017). Engel et al. (2013) conducted a study wherein they sought to assess the different resident gut microbiome across the lepidoptera species. It was observed that the proposed method of transmission of insect gut communities might be through maternal egg smearing, social transmission, acquisition from food and from the environment (Engel and Moran, 2013). It is clear from these studies that the gut microbiome of *M. sexta* might be derived both from environmental sources and from food-borne sources. However, during my study a key study was published by Hammer et al. (2017), who attempted to demonstrate that *Manduca sexta* and caterpillars lack a resident gut microbiome (Hammer et al., 2017). It studied the microbial load in a wide range of wild caterpillars reporting that bacteria number was very low in comparison to many other animals, and that all identified bacteria were derived from the leaves where these larvae were fed during their study. It was reported that the gut bacteria played no role in the host as caterpillars in which bacteria were suppressed demonstrated no difference in the weight of pupae, or time to pupation, compared to controls.

This study raises important points, and greatly extends the understanding of the caterpillar gut microbiome. However, it is not correct to state that caterpillars lack a gut microbiome. While the numbers of microbes present in the caterpillar gut is tens of thousands of times less than in many other animals, these contain very high numbers of bacteria ($>10^9$) and so tens of thousands of times less is still a significant number of bacteria (Engel and Moran, 2013). While the gut bacteria of caterpillars are derived from their environment, this is true of many animals with more complex microbiota, and the very long-standing evolutionary relationship between the Lepidoptera and their plant foods suggests it is likely that the Lepidoptera have evolved while being colonised by plant-

derived bacteria, that have shaped the caterpillar evolution (Voirol et al., 2018). In support of this, while there is variation between the gut microbiomes of the Lepidoptera, some core bacterial groups have emerged, which may indicate adaptation of both bacteria and host to each other. The study used antibiotics to supress gut bacteria, but a relatively narrow spectrum was used which likely suppressed but did not indeed eliminate the bacterial flora, and thus would not affect all bacteria. It is not clear how effective the antibacterial was, as the antibiotics were added to water which was sprayed onto the leaves where caterpillars were fed on.

Findings made in this thesis both agree and disagree with this profile of caterpillar gut microbiome. They support that relatively low number of bacteria inhabit the gut of caterpillars and these bacteria are derived from their environment. However, my findings disagree with the gut bacteria having no role in the growth and development of larvae. I have developed a protocol to raise bacteria-free larvae, using sterilisation of egg, sterile food and use of barrier conditions to generate germ-free *Manduca sexta* larvae. This is different to previous studies that have used antibiotics to suppress bacteria as a proxy for sterility. However, using this approach, a clear effect of removing or introducing colony foodborne bacteria and environment during pre-maturation (day 8) demonstrated that the absence of bacteria plays a role on the larval growth. Bacteria-free larvae display significantly reduced weight gain, particularly during the 5^{th} instar stage of development. The use of antibiotics, but here is a cocktail of antibiotics that was actively against a very broad range of bacteria, also resulted in poor growth of larvae, supporting this finding. Interestingly, the comparison between larvae in which bacterial growth was supressed by antibiotics still encountered bacteria, and sterile larvae that were raised in bacteria-free

environment revealed a difference in growth characteristics when viable bacteria were introduced during the later stages of growth. This suggests a role for exposure to bacteria in early growth and development, beyond just aiding in digestion of food materials. In conclusion, the gut microbiome has a major role in the growth of Bath colony *M. sexta*. Along with the ability to raise sterile larvae means that the use of *M. sexta* as a model for aspects of microbiome research should be further developed.

6.1 Further studies

Further studies are needed to validate the findings in this thesis. Several technical difficulties were encountered in terms of the *in vitro* culturing of the gut bacteria derived from the larvae of *Manduca sexta*. A low sequence reads were additionally obtained from certain samples when 16S rRNA sequencing was utilized to identify the type of bacteria genera present in the gut of the larvae of *Manduca sexta*. Indeed, to validate these findings, the use of whole genome sequencing technology (WGS) might provide a wider coverage and permit the identification of more bacterial species which might not have been detected using the 16S rRNA sequencing technologies. For the newly reported bacteria species and that includes differences in 17 bp and exhibits 97.69% 16S rRNA gene sequence similarity with *Oceanobacillus massiliensis* sp., further phenotypic characterizations should be conducted such as Gram staining, GC content, membrane lipid contents and the tolerance of carbohydrates assimilation in order to fully describe such novel bacterial species. In terms of the functional role of the gut microbiome in the immune system of the larvae, the gut morphological characteristics of larvae reared in sterile conditions should be assessed to determine if the absence of resident gut microbiome might lead to the incomplete

development of the gut of the larvae using similar methods described by Habineza *et al.* (2019). The latter author indeed used germ-free insect eggs to conduct a gnotobiotic experiment and thus to closely elucidate the role of certain gut microbiome residents of red palm pest *Rhynchophorus ferrugineus* Olivier on nutrients availability in the haemolymph. Thus, it is worth to carry out similar gnotobiotic studies to investigate the role of the bacteria were already isolated from Bath colony larvae on the maintenance of normal host physiology and development. Also, the presence or absence of immune cells such as haemocytes in the gut of the larvae should be elucidated as this will be key in determining if the absence of a resident gut microbiome has an effect on the development of the immune cells. The effect of bacterial exposure during the early days of larval growth should be studied. This could involve exposing sterile larvae to dead bacteria, specific bacterial components (peptidoglycan, LPS, lipoproteins) and specific types of bacteria and investigating the effect of priming of later growth.

Bibliography

Ahmad, I. M., Waldbauer, G. P. and Friedman, S. (1989) 'A defined artificial diet for the larvae of Manduca sexta', *Entomologia Experimentalis et Applicata*, 53(2), pp. 189–191. doi: 10.1111/j.1570-74581989.tb0303x.

Ahmed, I. *et al.* (2014) 'Description of lysinibacillus pakistanensis', *International Journal of Agriculture and Biology*, 16(2), pp. 447–450.

Albert, R. A. *et al.* (2007) 'Proposal of Viridibacillus gen. nov. and reclassification of Bacillus arvi, Bacillus arenosi and Bacillus neidei as Viridibacillus arvi gen. nov., comb. nov., Viridibacillus arenosi comb. nov. and Viridibacillus neidei comb. nov', *International Journal of Systematic and Evolutionary Microbiology*, 57(12), pp. 2729–2737. doi: 10.1099/ijs.0.65256-0.

Allen, H.K., Cloud-Hansen, K.A., Wolinski, J.M., Guan, C., Greene, S., Lu, S., Boeyink M., Broderick, N.A., Raffa, K.F. and Handelsman, J. 2009. Resident microbiota of the gypsy moth midgut harbors antibiotic resistance determinants. DNA Cell Biology, 28(3), pp. 109- 117.

Altschul, S. F. et al. (1990) 'Basic local alignment search tool', Journal of Molecular Biology, 215(3), pp. 403–410. doi: 10.1016/S0022-2836(05)80360-2. American Society for Microbiology, 17(4), pp. 840–862. doi: 10.1128/CMR.17.4.840-862.2004.

Anderson, K.E., Sheehan, T.H., Mott, B.M., Maes, P., Snyder, L., Schwan, M.R., Walton, A., Jones, B.M. and Corby-Harris, V. 2013. Microbial ecology of the hive and pollination landscape: bacterial associates from floral nectar, the alimentary tract and stored food of honey bees (Apis mellifera). PLoS One, 8:e83125.

Anjum, S. I. *et al.* (2018) 'Characterization of gut bacterial flora of Apis mellifera from north-west Pakistan', *Saudi Journal of Biological Sciences*. King Saud University, 25(2), pp. 388–392.doi:10.1016/j.sjbs.2017.05.008.Availableat:https://www.ncbi.nlm.nih.gov/pubmed/8177168.

Baldo, L. *et al.* (2019) 'Phylogeography and Ecological Niche Shape the Cichlid Fish Gut Microbiota in Central American and African Lakes', *Frontiers in Microbiology*, 10(October), pp. 1–19. doi: 10.3389/fmicb.2019.02372.

Ballhorn, D.J., Kautz, S., Lion, U. and Heil, M. 2008. Trade-offs between direct and indirect defences of lima bean (Phaseolus lunatus). Journal of Ecology, 96, pp. 971-980.

Balzola, F., Bernstein, C. and Van Assche, G. (2010) 'Nod2 is required for the regulation of commensal microbiota in the intestine: Commentary', *Inflammatory Bowel Disease Monitor*, 10(3), pp. 100–101.

Basu, S. *et al.* (2015) 'Evolution of bacterial and fungal growth media', *Bioinformation*, 11(4), pp. 182–184. doi: 10.6026/97320630011182.

Baumann, P., Moran, N., & Baumann, L. (1997). The Evolution and Genetics of Aphid Endosymbionts. *BioScience, 47*(1), 12-20. doi:10.2307/1313002

Beard, C. B., Cordon-Rosales, C., & Durvasula, R. V. (2002). Bacterial symbionts of the triatominae and their potential use in control of Chagas disease transmission. *Annual review of entomology*, *47*, 123–141. https://doi.org/10.1146/annurev.ento.47.091201.145144

Bevins, C. I., Martin-Porter, E. and Ganz, T. (1999) 'Defensins and innate host defence of the gastrointestinal tract', Gut, 45(6), pp. 911–915. doi: 10.1136/gut.45.6.911.

Bilen, M. et al. (2018) 'The contribution of culturomics to the repertoire of isolated human bacterial and archaeal species', Microbiome. Microbiome, 6(1), p. 94. doi: 10.1186/s40168-018- 0485-5.

Biron, D. G., Bonhomme, L., Coulon, M. and Øverli, Ø. 2014. Microbiomes, plausible players or not in alteration of host behaviour. Frontiers in Microbiology, 5, pp. 1-5.

Blaser, M. J. (2014) 'The microbiome revolution', *The Journal of Clinical Investigation*. The American Society for Clinical Investigation, 124(10), pp. 4162–4165. doi: 10.1172/JCI78366.

Bordenstein, S. R. and Theis, K. R. 2015. Host biology in light of the microbiome: Ten principles of holobionts and hologenomes. PLoS Biology,13, pp.1-23.

Brinkman, B. M. *et al.* (2011) 'Caspase deficiency alters the murine gut microbiome', *Cell Death and Disease*. Nature Publishing Group, 2(10), pp. 1–6. doi: 10.1038/cddis.2011.101.

Brinkmann, N., Martens, R. and Tebbe, C. C. 2008. Origin and diversity of metabolically active gut bacteria from laboratory-bred larvae of Manduca sexta (Sphingidae, Lepidoptera, Insecta). Applied and Environmental Microbiology, 74(23), pp. 7189-7196.

Broderick, N. A. and Lemaitre, B. (2012). Gut-associated microbes of Drosophila melanogaster. *Gut microbes*, *3*(4), 307–321. https://doi.org/10.4161/gmic.19896

Broderick, N. A. *et al.* (2004) 'Census of the Bacterial Community of the Gypsy Moth Larval Midgut by Using Culturing and Culture-Independent Methods', *Applied and Environmental Microbiology*, 70(1), pp. 293–300. doi: 10.1128/AEM.70.1.293-300.2004.
Broderick, N. A. *et al.* (2009) 'Contributions of gut bacteria to Bacillus thuringiensis-induced mortality vary across a range of Lepidoptera', *BMC Biology*, 7, pp. 1–9. doi: 10.1186/1741-7007-7-11.

Broderick, N. A., Raffa, K. F. and Handelsman, J. (2006) 'Midgut bacteria required for Bacillus thuringiensis insecticidal activity', Proceedings of the National Academy of Sciences of the United States of America, 103(41), pp. 15196–15199. doi: 10.1073/pnas.0604865103.

Brooks, A. W. *et al.* (2016) 'Phylosymbiosis: Relationships and Functional Effects of Microbial Communities across Host Evolutionary History', *PLoS Biology*, 14(11), pp. 1–29. doi: 10.1371/journal.pbio.2000225.

Brown, J. W. (no date) 'Molecular phylogenetic analysis using ribosomal RNA (rRNA)', pp. 1–14.

Browne, H. et al. 2016. Culturing of "unculturable" microbiota reveals novel taxa and extensive sporulation. Nature. 533: 543–546. pmid:27144353.

Brundage, A. L., Crippen, T. L. and Tomberlin, J. K. (2016) 'Methods for external disinfection of blow fly (Diptera: Calliphoridae) eggs prior to use in wound debridement therapy', *Wound Repair and Regeneration*, 24(2), pp. 384–393. doi: 10.1111/wrr.12408.

Brune, A. and Dietrich, C. (2015). The Gut Microbiota of Termites: Digesting the Diversity in the Light of Ecology and Evolution. *Annual review of microbiology*, *69*, 145–166. https://doi.org/10.1146/annurev-micro-092412-155715

Bukin, Y. S. et al. (2019) 'The effect of 16s rRNA region choice on bacterial community metabarcoding results', Scientific Data. The Author(s), 6, pp. 1–14. doi: 10.1038/sdata.2019.7.

Caminero, A. *et al.* (2014) 'Diversity of the cultivable human gut microbiome involved in gluten metabolism: Isolation of microorganisms with potential interest for coeliac disease', *FEMS Microbiology Ecology*, 88(2), pp. 309–319. doi: 10.1111/1574-6941.12295.

Caporaso, J. G. et al. (2012) 'Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms', ISME Journal. Nature Publishing Group, 6(8), pp. 1621– 1624. doi: 10.1038/ismej.2012.8.

Chakravorty, S. et al. (2007) ‘A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria’, Journal of microbiological methods. 2007/02/22, 69(2), pp. 330–339. doi: 10.1016/j.mimet.2007.02.005.

Chandler, J. A. *et al.* (2011) ‘Bacterial communities of diverse Drosophila species: Ecological context of a host-microbe model system’, *PLoS Genetics*, 7(9). doi: 10.1371/journal.pgen.1002272.

Chapman, R. F. (2013) *The Insects Structure and Function. 5th edition, edited by Stephen J. Simpson, Angela E. Douglas.*

Charroux, B. and Royet, J. (2012) ‘Gut-microbiota interactions in non-mammals: What can we learn from Drosophila?’, *Seminars in Immunology*. Elsevier Ltd, 24(1), pp. 17–24. doi: 10.1016/j.smim.2011.11.003.

Chen, B. *et al.* (2016) ‘Biodiversity and Activity of the Gut Microbiota across the Life History of the Insect Herbivore Spodoptera littoralis’, *Scientific Reports*. Nature Publishing Group, 6(January), pp. 1–14. doi: 10.1038/srep29505.

Chen, Z. et al. (2019) ‘Impact of Preservation Method and 16S rRNA Hypervariable Region on Gut Microbiota Profiling’, mSystems. Edited by J. G. Caporaso, 4(1), pp. e00271-18. doi: 10.1128/mSystems.00271-18.

Christensen, H., 2018. *Introduction To Bioinformatics In Microbiology*. Cham: Springer International Publishing.

Chun, J. and Rainey, F. A. (2014) ‘Integrating genomics into the taxonomy and systematics of the Bacteria and Archaea’, *International Journal of Systematic and Evolutionary Microbiology*, 64(PART 2), pp. 316–324. doi: 10.1099/ijs.0.054171-0.

Chun, J. et al. (2007) ‘EzTaxon: A web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences’, International Journal of Systematic and Evolutionary Microbiology, 57(10), pp. 2259–2261. doi: 10.1099/ijs.0.64915-0.

Chun, J. et al. (2018) ‘Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes’, International Journal of Systematic and Evolutionary Microbiology, 68(1), pp. 461–466. doi: 10.1099/ijsem.0.002516.

Clarridge, J. E. (2004) ‘Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases’, *Clinical Microbiology Reviews*, 17(4), pp. 840–862. doi: 10.1128/CMR.17.4.840-862.2004.

Clayton, A. M., Dong, Y. and Dimopoulos, G. (2014) ‘The anopheles innate immune system in the defense against malaria infection’, *Journal of Innate Immunity*, 6(2), pp. 169–181. doi: 10.1159/000353602.

Colman, D.R., Toolson, E.C., Takacs-Vesbach, C.D.. 2012. Do diet and taxonomy influence insect gut bacterial communities? Molecular Ecology, 21, pp. 5124-5137.

Cooper, D. and Eleftherianos, I. (2017) ‘Memory and specificity in the insect immune system: Current perspectives and future challenges’, *Frontiers in Immunology*, 8(MAY). doi: 10.3389/fimmu.2017.00539.

Corby-Harris, V. *et al.* (2007) ‘Geographical distribution and diversity of bacteria associated with natural populations of Drosophila melanogaster’, *Applied and Environmental Microbiology*, 73(11), pp. 3470–3479. doi: 10.1128/AEM.02120-06.

Cross, M. L. (2002) ‘Microbes versus microbes: Immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens’, *FEMS Immunology and Medical Microbiology*, 34(4), pp. 245–253. doi: 10.1016/S0928-8244(02)00377-2.

Da Silva, K.R.A., Rabinovitch, L. and Seldin, L. 1999. Phenotypic and genetic diversity among Bacillus sphaericus strains isolated in Brazil, potentially useful as biological control agents against mosquito larvae. Research Microbiology, 150, pp. 153-160.

Daffonchio, D., Borin, S., Frova, G., Manachini, P.L. and Sorlini, C. 1998. PCR fingerprinting of whole genomes: the spacers between the 16S and 23S rRNA genes and of intergenictRNA gene regions reveal a different intraspecific genomic variability of Bacillus cereus and Bacillus licheniformis. International Journal of System Bacteriology, 48, pp. 107-116.

Dalal, S. R. and Chang, E. B. 2014. The microbial basis of inflammatory bowel diseases. Journal of Clinical Investigation, 124(10), pp. 4190-4196.

Dame, N. (1960) 'The Germ-free Animal', (10). New series, Vol. 5, No. 10.

Daniel E Shumer, N. J. N. N. P. S. (2017) '乳鼠心肌提取 HHS Public Access', *Physiology & behavior*, 176(12), pp. 139–148. doi: 10.1038/nm.4517.Current.

De Clerck, E. and De Vos, P., 2004. Genotypic diversity among Bacillus licheniformis strain from various sources. FEMS Microbiology Letters, 231, pp. 91-98

De Vooght, L. *et al.* (2015) 'Paternal transmission of a secondary symbiont during mating in the viviparous tsetse fly', *Molecular Biology and Evolution*, 32(8), pp. 1977–1980. doi: 10.1093/molbev/msv077.

Desai, M. S. and Brune, A. (2012) 'Bacteroidales ectosymbionts of gut flagellates shape the nitrogen-fixing community in dry-wood termites', *ISME Journal*. Nature Publishing Group, 6(7), pp. 1302–1313. doi: 10.1038/ismej.2011.194.

DeSantis, T. Z. et al. (2006) 'Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB', Applied and environmental

microbiology. American Society for Microbiology, 72(7), pp. 5069–5072. doi: 10.1128/AEM.03006-05.

Dillon, R. J. *et al.* (2008) 'Composition of Acridid gut bacterial communities as revealed by 16S rRNA gene analysis', *Journal of Invertebrate Pathology*, 97(3), pp. 265–272. doi: 10.1016/j.jip.2007.09.010.

Dillon, R. J. *et al.* (2010) 'Diversity of gut microbiota increases with aging and starvation in the desert locust', *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, 97(1), pp. 69–77. doi: 10.1007/s10482-009-9389-5.

Dionne, M. S. and Schneider, D. S. (2008) 'Models of infectious diseases in the fruit fly Drosophila melanogaster', *DMM Disease Models and Mechanisms*, 1(1), pp. 43–49. doi: 10.1242/dmm.000307.

Douglas, A. E. (2015). Multiorganismal insects: diversity and function of resident microorganisms. Annual Review of Entomology, 60, 17-34.

Dow (1992) 'pH GRADIENTS IN LEPIDOPTERAN MIDGUT.', *The Journal of experimental biology*, 172(Pt 1), pp. 355–375. doi: 10.1271/bbb1961.35.1154.

Ehling-Schulz, M., Lereclus, D. and Koehler, T. M. (2019) *The Bacillus cereus Group: Bacillus Species with Pathogenic Potential*, *Microbiology Spectrum*. doi: 10.1128/microbiolspec.gpp3-0032-2018.

Eichler, S. and Schaub, G. A. (2002) 'Development of symbionts in triatomine bugs and the effects of infections with trypanosomatids', *Experimental Parasitology*, 100(1), pp. 17–27. doi: 10.1006/expr.2001.4653.

Eisenhofer, R. et al. (2019) 'Contamination in Low Microbial Biomass Microbiome Studies: Issues and Recommendations', Trends in Microbiology. Elsevier, 27(2), pp. 105–117. doi: 10.1016/j.tim.2018.11.003.

Eleftherianos, I., R. H. ffrench-Constant, et al. (2010). "Dissecting the immune response to the entomopathogen Photorhabdus." Trends Microbiol 18: 552-560.

Elinav, E. *et al.* (2011) 'NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis', *Cell*, 145(5), pp. 745–757. doi: 10.1016/j.cell.2011.04.022.

Engel, P. and Moran, N. A. (2013) 'The gut microbiota of insects – diversity in structure and function', FEMS Microbiology Reviews. John Wiley & Sons, Ltd (10.1111), 37(5), pp. 699–735. doi: 10.1111/1574-6976.12025.

Engel, P., Martinson, V.G., Moran, N.A. 2012. Functional diversity within the simple gut microbiota of the honey bee. Proceedings of the National Academy of Sciences of the United States of America, 109, pp. 11002-11007.

Fiedorová, K. *et al.* (2019) 'The impact of DNA extraction methods on stool bacterial and fungal microbiota community recovery', *Frontiers in Physiology*, 10(April), pp. 1–11. doi: 10.3389/fmicb.2019.00821.

Fouhy, F. *et al.* (2016) '16S rRNA gene sequencing of mock microbial populations-impact of DNA extraction method, primer choice and sequencing platform', *j*. Springer Nature, 16(1). doi: 10.1186/s12866-016-0738-z.

Fraher, M., O'Toole, P. and Quigley, E. (2012) 'Techniques used to characterize the gut microbiota: A guide for the clinician', *Nature reviews. Gastroenterology & hepatology*, 9, pp. 312–322. doi: 10.1038/nrgastro.2012.44.

Friedman, M. 2002. Tomato glycoalkaloids: Role in the plant and in the diet. Journal of Agriculture and Food Chemistry, 50, pp. 5751-5780.

Fu, R. *et al.* (2020) 'Comparing the intestinal bacterial communies of sympatric wintering Hooded Crane (Grus monacha) and Domestic Goose (Anser anser domesticus)', *Avian Research*. BioMed Central, 11(1), pp. 1–9. doi: 10.1186/s40657-020-00195-9.

Fukatsu, T. and Hosokawa, T. (2002) ‘Capsule-transmitted gut symbiotic bacterium of the Japanese common plataspid stinkbug, megacopta punctatissima’, *Applied and Environmental Microbiology*, 68(1), pp. 389–396. doi: 10.1128/AEM.68.1.389-396.2002.

Garud, N. R. *et al.* (2019) ‘Evolutionary dynamics of bacteria in the gut microbiome within and across hosts’, *PLoS Biology*, 17(1), pp. 1–29. doi: 10.1371/JOURNAL.PBIO.3000102.

Genersch, E. (2010) ‘American Foulbrood in honeybees and its causative agent, Paenibacillus larvae’, *Journal of Invertebrate Pathology*. Elsevier Inc., 103(SUPPL. 1), pp. S10–S19. doi: 10.1016/j.jip.2009.06.015.

Goharrostami, M. and Jalali Sendi, J. (2018) ‘Investigation on endosymbionts of Mediterranean flour moth gut and studying their role in physiology and biology’, *Journal of Stored Products Research*. Elsevier Ltd, 75, pp. 10–17. doi: 10.1016/j.jspr.2017.11.003.

Gottlieb, Y. *et al.* (2006) ‘Identification and localization of a Rickettsia sp. in Bemisia tabaci (Homoptera: Aleyrodidae)’, *Applied and Environmental Microbiology*, 72(5), pp. 3646–3652. doi: 10.1128/AEM.72.5.3646-3652.2006.

Gouin, A. *et al.* (2017) ‘Two genomes of highly polyphagous lepidopteran pests (Spodoptera frugiperda, Noctuidae) with different host-plant ranges’, *Scientific Reports*, 7(1), pp. 1–12. doi: 10.1038/s41598-017-10461-4.

Govind, S. (2008) ‘Innate immunity in Drosophila: Pathogens and pathways’, *Insect Science*, 15(1), pp. 29–43. doi: 10.1111/j.1744-7917.2008.00185.x.

Grabowski, N. T. and Klein, G. (2017) ‘Microbiology of processed edible insect products – Results of a preliminary survey’, *International Journal of Food Microbiology*. Elsevier B.V., 243, pp. 103–107. doi: 10.1016/j.ijfoodmicro.2016.11.005.

Grady, E. N. *et al.* (2016) 'Current knowledge and perspectives of Paenibacillus: A review', *Microbial Cell Factories*. BioMed Central, 15(1), pp. 1–18. doi: 10.1186/s12934-016-0603-7.

Greub, G. (2012) 'Culturomics: A new approach to study the human microbiome', *Clinical Microbiology and Infection,* 18(12), pp. 1157–1159. doi: 10.1111/1469-0691.12032.

Grigorescu, A. S. et al. (2018) 'Accessing the Hidden Microbial Diversity of Aphids : an Illustration of How Culture-Dependent Methods Can Be Used to Decipher the Insect Microbiota'. Microbial Ecology, pp. 1035–1048.

Grunert, L. W. *et al.* (2015) 'A quantitative analysis of growth and size regulation in manduca sexta: The physiological basis of variation in size and age at metamorphosis', *PLoS ONE,* 10(5). doi: 10.1371/journal.pone.0127988.

Gunaratna, R. and Jiang, H. 2013. A comprehensive analysis of the Manduca sexta immunotranscriptome. Developmental and Comparative Immunology, 39(4), pp.388-398.

Gupta, A. and Nair, S. (2020) 'Dynamics of Insect–Microbiome Interaction Influence Host and Microbial Symbiont', *Frontiers in Microbiology,* 11(June). doi: 10.3389/fmicb.2020.01357.

Gurung, K., Wertheim, B. and Falcao Salles, J. (2019) 'The microbiome of pest insects: it is not just bacteria', Entomologia Experimentalis et Applicata. John Wiley & Sons, Ltd (10.1111), 167(3), pp. 156–170. doi: 10.1111/eea.12768.

Gutell, R. R., Larsen, N. and Woese, C. R. (1994) 'Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective', Microbiological reviews, 58(1), pp. 10–26.

Habineza, P. *et al.* (2019) 'The Promoting Effect of Gut Microbiota on Growth and Development of Red Palm Weevil, Rhynchophorus ferrugineus (Olivier) (Coleoptera: Dryophthoridae) by modulating its nutritional metabolism', *Frontiers in Microbiology*, 10(MAY), pp. 1–10. doi: 10.3389/fmicb.2019.01212.

Hammer, T. J. et al. (2017) 'Caterpillars lack a resident gut microbiome', Proceedings of the National Academy of Sciences of the United States of America, 114(36), pp. 9641–9646. doi: 10.1073/pnas.1707186114.

Hammer, T.J., McMillan, W.O. and Fierer, N. 2104. Metamorphosis of a Butterfly-Associated Bacterial Community. PLOS One 9 (1):e86995.

Hansen, A. K., & Moran, N. A. (2014). The impact of microbial symbionts on host plant utilization by herbivorous insects. *Molecular ecology*, *23*(6), 1473–1496. https://doi.org/10.1111/mec.12421

Harada, H. and Ishikawa, H. (1997) 'Experimental pathogenicity of Erwinia aphidicola to pea aphid, Acyrthosiphon pisum', *Journal of General and Applied Microbiology*, 43(6), pp. 363–367. doi: 10.2323/jgam.43.363.

Harrison, J. F. (2001) 'I NSECT A CID -B ASE P HYSIOLOGY'. Annual reviews. 2001. 46:221–50

Hemmi, H. *et al.* (2000) 'A Toll-like receptor recognizes bacterial DNA', *Nature*, 408(6813), pp. 740–745. doi: 10.1038/35047123.

Herman, L. and Heyndrickx, M. 2000. The presence of intragenically located REP-like elements in Bacillus sporothermodurans is sufficient for REP-PCR typing. Research.

Heyrman, J. *et al.* (2005) 'Bacillus arenosi sp. nov., Bacillus arvi sp. nov. and Bacillus humi sp. nov., isolated from soil', *International Journal of Systematic and Evolutionary Microbiology*, 55(1), pp. 111–117. doi: 10.1099/ijs.0.63240-0.

Hill, D. *et al.* (2018) 'The Lactobacillus casei group: History and health related applications', *Frontiers in Microbiology*, 9(SEP), pp. 1–12. doi: 10.3389/fmicb.2018.02107.

Hongoh, Y. *et al.* (2005) 'Intra- and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host', *Applied and Environmental Microbiology*, 71(11), pp. 6590–6599. doi: 10.1128/AEM.71.11.6590-6599.2005.

Hongoh, Y. *et al.* (2008) 'Genome of an endosymbiont coupling N2 fixation to cellulolysis within protist cells in termite gut', *Science*, 322(5904), pp. 1108–1109. doi: 10.1126/science.1165578.

Hosokawa, T. *et al.* (2006) 'Strict host-symbiont cospeciation and reductive genome evolution in insect gut bacteria', *PLoS Biology*, 4(10), pp. 1841–1851. doi: 10.1371/journal.pbio.0040337.

http://omegabioservices.com/index.php/16s-reference/ (Accessed 17 December 2020).

https://www.hgsc.bcm.edu/tobacco-hornworm-genome-project
(Accessed date 24 May 2017).

Hurst, M. R. H., Glare, T. R. and Jackson, T. A. (2004) 'Erratum: Cloning Serratia entomophila antifeeding genes - A putative defective prophage active against the grass grub Costelytra zealandica (Journal of Bacteriology (2004) 186, 15 (5116-5128))', *Journal of Bacteriology*, 186(20), pp. 7023–7024. doi: 10.1128/JB.186.20.7023-7024.2004.

Hussa, E. and Goodrich-Blair, H. (2012) ‘Rearing and Injection of Manduca sexta Larvae to Assess Bacterial Virulence’, Journal of Visualized Experiments, (70), pp. 1–5. doi: 10.3791/4295.

Indiragandhi, P. *et al.* (2008) ‘Characterization of plant growth-promoting traits of bacteria isolated from larval guts of Diamondback moth Plutella xylostella (Lepidoptera: Plutellidae)’, *Current Microbiology*, 56(4), pp. 327–333. doi: 10.1007/s00284-007-9086-4.

Ito, T. *et al.* (2019) ‘Conventional culture methods with commercially available media unveil the presence of novel culturable bacteria’, *Gut Microbes*. Taylor & Francis, 10(1), pp. 77–91. doi: 10.1080/19490976.2018.1491265.

Janda, J. M. and Abbott, S. L. (2007) ‘16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls’, Journal of Clinical Microbiology, 45(9), pp. 2761–2764. doi: 10.1128/JCM.01228-07.

Jernberg, C., Löfmark, S., Edlund, C. and Jansson, J.K. Long-term impacts of antibiotic exposure on the human intestinal microbiota. 2010. Microbiology, 156, pp. 3216-3223.

Jiang, L. *et al.* (2019) ‘Distinct Functions of Bombyx mori Peptidoglycan Recognition Protein 2 in Immune Responses to Bacteria and Viruses’, *Frontiers in Immunology*, 10(APR), pp. 1–14. doi: 10.3389/fimmu.2019.00776.

Johnson, J. S. *et al.* (2019) ‘Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis’, *Nature Communications*. Springer US, 10(1), pp. 1–11. doi: 10.1038/s41467-019-13036-1.

Kanost, M. R., Jiang, H. and Yu, X. Q. (2004) ‘Innate immune responses of a lepidopteran insect, Manduca sexta’, Immunological Reviews, 198, pp. 97–105. doi: 10.1111/j.0105- 2896.2004.0121.x.

Kaufman, Michael G, Klug, Michael J & Merritt, Richard W, 1989. Growth and food utilization parameters of germ-free house crickets, Acheta domesticus. Journal of insect physiology: including endocrinology, neurobiology, pharmacology, nutrition, reproductive biology., 35(12), pp.957–967.

Kelsall, B. L. and Leon, F. (2005) 'Involvement of intestinal dendritic cells in oral tolerance, immunity to pathogens, and inflammatory bowel disease', *Immunological Reviews*, 206, pp. 132–148. doi: 10.1111/j.0105-2896.2005.00292.x.

Kennedy, E. A., King, K. Y. and Baldridge, M. T. (2018) 'Mouse microbiota models: Comparing germ-free mice and antibiotics treatment as tools for modifying gut bacteria', *Frontiers in Physiology*, 9(OCT), pp. 1–16. doi: 10.3389/fphys.2018.01534.

Kikuchi, Y. *et al.* (2012) 'Symbiont-mediated insecticide resistance', *Proceedings of the National Academy of Sciences of the United States of America*, 109(22), pp. 8618–8622. doi: 10.1073/pnas.1200231109.

Kikuchi, Y., Hosokawa, T. and Fukatsu, T. (2007) 'Insect-microbe mutualism without vertical transmission: A stinkbug acquires a beneficial gut symbiont from the environment every generation', *Applied and Environmental Microbiology*, 73(13), pp. 4308–4316. doi: 10.1128/AEM.00067-07.

Kim, D. *et al.* (2017) 'Optimizing methods and dodging pitfalls in microbiome research', *Microbiome*. Microbiome, 5(1), pp. 1–14. doi: 10.1186/s40168-017-0267-5.

Kim, M. et al. (2014) 'Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes', International Journal of Systematic and Evolutionary Microbiology, 64(PART 2), pp. 346–351. doi: 10.1099/ijs.0.059774-0.

Kim, O. et al. 2012. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol 3:716-21.

Kim, Y.J., Choi, J.H., Kim, J.Y., Shin, N.R., Kim, S.H., Lee,W.J. and Bae, J.W. 2014. Insect gut bacterial diversity determined by environmental habitat, diet, developmental stage, and phylogeny of host. Applied and Environmental Microbioliology, 80(17), pp. 5254-5264.

Kingsolver, J. G. (2007) 'Variation in growth and instar number in field and laboratory Manduca sexta', Proceedings of the Royal Society B: Biological Sciences, 274(1612), pp. 977–981. doi: 10.1098/rspb.2006.0036.

Koch, H. and Schmid-Hempel, P. (2011) 'Socially transmitted gut microbiota protect bumble bees against an intestinal parasite', *Proceedings of the National Academy of Sciences of the United States of America*, 108(48), pp. 19288–19292. doi: 10.1073/pnas.1110474108.

Köhler, T. *et al.* (2012) 'High-resolution analysis of gut environment and bacterial microbiota reveals functional compartmentation of the gut in wood-feeding higher termites (Nasutitermes spp.)', *Applied and Environmental Microbiology*, 78(13), pp. 4691–4701. doi: 10.1128/AEM.00683-12.

Kölsch, G. and Pedersen, B. V (2010) 'Can the tight co-speciation between reed beetles (Col., Chrysomelidae, Donaciinae) and their bacterial endosymbionts, which provide cocoon material, clarify the deeper phylogeny of the hosts?', *Molecular phylogenetics and evolution*. Elsevier, 54(3), pp. 810–821.

Kölsch, G., Matz-Grund, C. and Pedersen, B. V (2009) 'Ultrastructural and molecular characterization of endosymbionts of the reed beetle genus Macroplea (Chrysomelidae, Donaciinae), and proposal of "Candidatus Macropleicola appendiculatae" and "Candidatus Macropleicola muticae"', *Canadian journal of microbiology*. NRC Research Press, 55(11), pp. 1250–1260.

Kostic, A. D., Howitt, M. R. and Garrett, W. S. (2013) 'Exploring host-microbiota interactions in animal models and humans', *Genes and Development*, 27(7), pp. 701–718. doi: 10.1101/gad.212522.112.

Krams, I. A. *et al.* (2017) 'Microbiome symbionts and diet diversity incur costs on the immune system of insect larvae', *Journal of Experimental Biology*, 220(22), pp. 4204–4212. doi: 10.1242/jeb.169227.

La Scola, B. *et al.* (2014) 'Aerobic culture of anaerobic bacteria using antioxidants: a preliminary report', *European Journal of Clinical Microbiology & Infectious Diseases*, 33(10), pp. 1781–1783. doi: 10.1007/s10096-014-2137-4.

Lagier, J. C. et al. (2018) 'Culturing the human microbiota and culturomics', Nature Reviews Microbiology. Springer US, 16(9), pp. 540–550. doi: 10.1038/s41579-018-0041-0.

Lagier, J.-C. *et al.* (2012) 'Microbial culturomics: paradigm shift in the human gut microbiome study', *Clinical Microbiology and Infection*. Elsevier, 18(12), pp. 1185–1193.

Lau, J. T. *et al.* (2016) 'Capturing the diversity of the human gut microbiota through culture-enriched molecular profiling', *Genome Medicine*. Genome Medicine, 8(1), pp. 1–10. doi: 10.1186/s13073-016-0327-7.

Le Page, S. *et al.* (2016) 'Noncontiguous finished genome sequence and description of Enterococcus massiliensis sp. nov.', *New Microbes and New Infections*, 12, pp. 90–95. doi: 10.1016/j.nmni.2016.04.011.

Lehane, M. J. (1997) 'Peritrophic matrix structure and function', *Annual Review of Entomology*, 42(100), pp. 525–550. doi: 10.1146/annurev.ento.42.1.525.

Lemke, T., et al. (2003). Physicochemical conditions and microbial activities in the highly alkaline gut of the humus-feeding larva of Pachnoda ephippiata (Coleoptera: Scarabaeidae). Applied & Environmental Microbiology, 69, 2003, p. 6650- 6658.

Leroy, P. D. *et al.* (2011) 'Microorganisms from aphid honeydew attract and enhance the efficacy of natural enemies. Nat Commun 2'.

Ley, R.E., Hamady, M., Lozupone, C., Turnbaugh, P.J., Ramey, R.R., Bircher, J.S., Schlegel, M.L., Tucker, T.A., Schrenzel, M.D., Knight, R. and Gordon, J.I. 2008. Evolution of mammals and their gut microbes. Science, 320, 1647-1651.

Littman, D. R. and Pamer, E. G. (2011) 'Role of the commensal microbiota in normal and pathogenic host immune responses', *Cell Host and Microbe*, 10(4), pp. 311–323. doi: 10.1016/j.chom.2011.10.004.

Liu, P.Y.F., Ke, S.C. and Chen, S.L. 1997. Use of pulsed-fields gel electrophoresis to investigate a pseudo-outbreak of Bacillus cereus in a pediatric unit. Journal of Clinical Microbiology, 35, 1533-1535.

Lu, J., Nogi, Y. and Takami, H. (2001) 'Oceanobacillus iheyensis gen. nov., sp. nov., a deep-sea extremely halotolerant and alkaliphilic species isolated from a depth of 1050 m on the Iheya Ridge', *FEMS Microbiology Letters*, 205(2), pp. 291–297. doi: 10.1016/S0378-1097(01)00493-1.

Luigi Franchi, Raul Muñoz-Planillo, and G. N. (2012) 'Sensing and Reacting to Microbes via the Inflammasomes'. doi: 10.1038/ni.2231.

Lundgren, J. G. and Lehman, R. M. (2010) 'Bacterial Gut Symbionts Contribute to Seed Digestion in an Omnivorous Beetle', PLoS ONE, 5(5). doi: 10.1371/journal.pone.0010831.

Mahar, A. N. *et al.* (2005) 'Pathogenicity of bacterium, Xenorhabdus nematophila isolated from entomopathogenic nematode (Steinernema carpocapsae) and its secretion against Galleria mellonella larvae', *Journal of Zhejiang University: Science*, 6 B(6), pp. 457–463. doi: 10.1631/jzus.2005.B0457.

Maldonado Galdeano, C. *et al.* (2019) 'Beneficial effects of probiotic consumption on the immune system', *Annals of Nutrition and Metabolism*, 74(2), pp. 115–124. doi: 10.1159/000496426.

Malla, M. A. et al. (2019) 'Exploring the human microbiome: The potential future role of next- generation sequencing in disease diagnosis and treatment', Frontiers in Immunology, 10(JAN), pp. 1–23. doi: 10.3389/fimmu.2018.02868.

Martens, E.C., Heungens, K, and Goodrich-Blair, H. 2003. Early colonization events in the mutualistic association between Steinernema carpocapsae nematodes and Xenorhabdus nematophila bacteria. Journal of Bacteriology, 185, pp. 3147-3154.

Martinson, V. G. *et al.* (2011) 'A simple and distinctive microbiota associated with honey bees and bumble bees', *Molecular Ecology*. Wiley Online Library, 20(3), pp. 619–628.

Mason, K.L., Stepien, T.A., Blum, J.E., Holt, J.F., Labbe, N.H., Rush, J.S., Raffa, K.F. and Handelsman, J. 2011. From commensal to pathogen: translocation of Enterococcus faecalis from the midgut to the hemocoel of Manduca sexta. MBio, 2(3), pp. e00065-11.

Mazmanian, S., Liu, C., Tzianabos, A. and Kasper, D. 2005. An Immunomodulatory Molecule of Symbiotic Bacteria Directs Maturation of the Host Immune System. Cell, 122(1), pp.107118.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V, Domazet-Lošo, T., Douglas, A. E., … Wernegreen, J. J. 2013. Animals in a bacterial world, a new imperative for the life sciences. Proceedings of the National Academy of Sciences, 110 (9), pp. 3229-3236.

McMillan, L. E. and Adamo, S. A. (2020) ‘Friend or foe? Effects of host immune activation on the gut microbiome in the caterpillar Manduca sexta ’, *The Journal of Experimental Biology*, 223(19), p. jeb226662. doi: 10.1242/jeb.226662.

Mendo, S.A.L.V., Henriques, S.I., Correria, A.C.M. and Duarte, J.M.C. 2000. Genetic characterisation of new thermo tolerant Bacillus licheniformis strain. Current Microbiology, 40, pp. 137-139. Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases.

Miller, W. J. (2013) ‘Bugs in Transition: The Dynamic World of Wolbachia in Insects’, *PLoS Genetics*, 9(12), pp. 12–13. doi: 10.1371/journal.pgen.1004069.

Mita, K. *et al.* (2004) ‘The Genome Sequence of Silkworm, Bombyx mori’, *DNA Research*, 11(1), pp. 27–35. doi: 10.1093/dnares/11.1.27.

Moll, R. M. *et al.* (2001) ‘Meconial peritrophic membranes and the fate of midgut bacteria during mosquito (Diptera: Culicidae) metamorphosis’, *Journal of Medical Entomology*, 38(1), pp. 29–32. doi: 10.1603/0022-2585-38.1.29.

Moran, N.A, McCutcheon, J.P. and Nakabachi, A. 2008. Genomics and evolution of heritable bacterial symbionts. Annual Review of Genetics, 42, pp.165-190.

Münch, A. *et al.* (2008) ‘Photorhabdus luminescens genes induced upon insect infection’, *BMC Genomics*, 9, pp. 1–17. doi: 10.1186/1471-2164-9-229.

Nagpal, R. *et al.* (2018) ‘Comparative microbiome signatures and short-chain fatty acids in mouse, rat, non-human primate, and human feces’, *Frontiers in Microbiology*, 9(NOV), pp. 1–13. doi: 10.3389/fmicb.2018.02897.

Nakajima, H. *et al.* (2005) 'Spatial distribution of bacterial phylotypes in the gut of the termite Reticulitermes speratus and the bacterial community colonizing the gut epithelium', *FEMS Microbiology Ecology*. Blackwell Publishing Ltd Oxford, UK, 54(2), pp. 247–255.

Näpflin, K. and Schmid-Hempel, P. (2016) 'Immune response and gut microbial community structure in bumblebees after microbiota transplants', *Proceedings of the Royal Society B: Biological Sciences*, 283(1831). doi: 10.1098/rspb.2016.0312.

Nehme, N. T. *et al.* (2007) 'A model of bacterial intestinal infections in Drosophila melanogaster', *PLoS Pathogens*, 3(11), pp. 1694–1709. doi: 10.1371/journal.ppat.0030173.

Nishiwaki, H. *et al.* (2007) 'Insecticidal bacteria isolated from predatory larvae of the antlion species Myrmeleon bore (Neuroptera: Myrmeleontidae)', *Journal of Invertebrate Pathology*, 96(1), pp. 80–88. doi: 10.1016/j.jip.2007.02.007.

Ozburn, R. H. (1940) "Bio-ecology. By F. E. Clement and V. E. Shelford. John Wiley & Sons, Inc., New York. Pages i—vi and 1—425; 85 figures. 1939. Price $4.50.," *The Canadian Entomologist*, Cambridge University Press, 72(5), pp. 108–108.

Pandya, S. *et al.* (2017) 'Comparison of culture-dependent and culture-independent molecular methods for characterization of vaginal microflora', *Journal of Medical Microbiology*, 66(2), pp. 149–153. doi: 10.1099/jmm.0.000407.

Pell, M., J. et al. (2006). Soil respiration. pp. 117-126. In: J. Bloem, D. W. Hopking, and A. Benedetti (eds.). Microbiological methods for assessing quality. CAB International. Wallingford, UK.

Pereira, F. C. and Berry, D. (2017) ‘Microbial nutrient niches in the gut’, *Environmental Microbiology*, 19(4), pp. 1366–1378. doi: 10.1111/1462-2920.13659.

Poretsky, R. et al. (2014) ‘Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics’, PloS one. Public Library of Science, 9(4), pp. e93827–e93827. doi: 10.1371/journal.pone.0093827.

Rakoff-Nahoum, S. *et al.* (2004) ‘Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis’, *Cell*, 118(2), pp. 229–241. doi: 10.1016/j.cell.2004.07.002.

Rawls, J. F. *et al.* (2006) ‘Reciprocal Gut Microbiota Transplants from Zebrafish and Mice to Germ-free Recipients Reveal Host Habitat Selection’, *Cell*, 127(2), pp. 423–433. doi: 10.1016/j.cell.2006.08.043.

Raymond, F. *et al.* (2019) ‘Culture-enriched human gut microbiomes reveal core and accessory resistance genes’, *Microbiome*. Microbiome, 7(1), pp. 1–13. doi: 10.1186/s40168-019-0669-7.

Rizzi, A. et al. (2013) ‘Characterization of the bacterial community associated with larvae and adults of anoplophora chinensis collected in Italy by culture and culture-independent methods’, BioMed Research International, 2013. doi: 10.1155/2013/420287.

Roe, A. D. *et al.* (2009) ‘Evolutionary framework for Lepidoptera model systems’, *Genetics and Molecular Biology of Lepidoptera. CRC Press, Boca Raton*, pp. 1–24.

Roeselers, G. *et al.* (2011) ‘Evidence for a core gut microbiota in the zebrafish’, *ISME Journal*. Nature Publishing Group, 5(10), pp. 1595–1608. doi: 10.1038/ismej.2011.38.

Rolff, J., Reynolds, S. and of London, R. E. S. (2009) *Insect Infection and Immunity: Evolution, Ecology, and Mechanisms*. OUP Oxford (Oxford biology). Available at: https://books.google.co.uk/books?id=kQYWDAAAQBAJ.

Round, J. L., & Mazmanian, S. K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. *Nature reviews. Immunology*, *9*(5), 313–323. https://doi.org/10.1038/nri2515

Round, J., Lee, S., Li, J., Tran, G., Jabri, B., Chatila, T. and Mazmanian, S. (2011). The TollLike Receptor 2 Pathway Establishes Colonization by a Commensal of the Human Microbiota.

Roux, V. *et al.* (2013) 'Non-contiguous finished genome sequence and description of Oceanobacillus massiliensis sp. nov', *Standards in genomic sciences*. Michigan State University, 9(2), pp. 370–384. doi: 10.4056/sigs.4267953.

Ruokolainen, L. et al. (2016) 'Larval growth rate is associated with the composition of the gut microbiota in the Glanville fritillary butterfly', Oecologia. Springer Berlin Heidelberg, 181(3), pp. 895–903. doi: 10.1007/s00442-016-3603-8. Science, 332(6032), pp.974-977.

Sabat, D. *et al.* (2015) 'A Protocol to Generate Germ Free Drosophila for Microbial Interaction Studies', *Advanced Techniques in Biology & Medicine*, s1, pp. 1–6. doi: 10.4172/2379-1764.s1-001.

Schilder, R. J. and Marden, J. H. (2007) 'Metabolic syndrome in insects triggered by gut microbes', *Journal of Diabetes Science and Technology*, 1(5), pp. 794–796. doi: 10.1177/193229680700100526.

Sears, C. L., Geis, A. L. and Housseau, F. 2014. Bacteroides fragilis subverts mucosal biology: From symbiont to colon carcinogenesis. Journal of Clinical Investigation, 124(10), pp. 41664172.

Sessitsch, A. et al. (2002) 'Cultivation-independent population analysis of bacterial endophytes in three potato varieties based on eubacterial and Actinomycetes-specific PCR of 16S rRNA genes', FEMS Microbiology Ecology, 39(1), pp. 23–32. doi: 10.1016/S0168-6496(01)00189-1.

Shao, Y. et al. (2017) 'Symbiont-Derived Antimicrobials Contribute to the Control of the Lepidopteran Gut Microbiota', Cell Chemical Biology. Elsevier Ltd., 24(1), pp. 66–75. doi: 10.1016/j.chembiol.2016.11.015.

Shao, Y., Spiteller, D., Tang, X., Ping, L., Colesie, C., Münchberg, U., Bartram, S., Schneider B., Büdel, B., Popp, J., Heckel, D.G. and Boland W. 2011. Crystallization of and b-carotene in the foregut of Spodoptera larvae feeding on a toxic food plant. Insect Biochemistry and Molecular Biology, 41, 273-281.

Sharma, M. and Shukla, G. (2016) 'Metabiotics: One step ahead of probiotics; an insight into mechanisms involved in anticancerous effect in colorectal cancer', Frontiers in Microbiology, 7(DEC), pp. 1–15. doi: 10.3389/fmicb.2016.01940.

Sharpton, T. J. (2018) 'Role of the Gut Microbiome in Vertebrate Evolution', *mSystems*, 3(2), pp. e00174-17. doi: 10.1128/msystems.00174-17.

Shin, S. C. *et al.* (2011) 'Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin signaling', *Science*, 334(6056), pp. 670–674. doi: 10.1126/science.1212782.

Singh, S. *et al.* (2014) 'Microbial population dynamics in the hemolymph of Manduca sexta infected with Xenorhabdus nematophila and the entomopathogenic nematode Steinernema carpocapsae', *Applied and environmental microbiology*. 2014/05/09. American Society for Microbiology, 80(14), pp. 4277–4285. doi: 10.1128/AEM.00768-14.

Sousa, A. M. et al. (2013) 'Improvements on colony morphology identification towards bacterial profiling', Journal of Microbiological Methods, 95(3), pp. 327–335. doi: 10.1016/j.mimet.2013.09.020.

Stappenbeck, T. S., Hooper, L. V. and Gordon, J. I. (2002) 'Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells', *Proceedings of the National Academy of Sciences of the United States of America*, 99(24), pp. 15451–15455. doi: 10.1073/pnas.202604299.

Staudacher, H. *et al.* (2016) 'Variability of bacterial communities in the moth Heliothis virescens indicates transient association with the host', *PLoS ONE*, 11(5), pp. 1–21. doi: 10.1371/journal.pone.0154514.

Stavrinides, J., McCloskey, J. K. and Ochman, H. (2009) 'Pea aphid as both host and vector for the phytopathogenic bacterium Pseudomonas syringae', *Applied and environmental microbiology*. Am Soc Microbiol, 75(7), pp. 2230–2235.

Stephens, W. Z. *et al.* (2016) 'The composition of the zebrafish intestinal microbial community varies across development', *ISME Journal*. Nature Publishing Group, 10(3), pp. 644–654. doi: 10.1038/ismej.2015.140.

Strathdee, F and Free, A. 2013. Denaturing gradient gel electrophoresis (DGGE). Methods in Molecular Biology, 1054, pp.145-157.

Su, C., Lei, L., Duan, Y., Zhang, K.Q. and Yang, J. 2012. Culture-independent methods for studying environmental microorganisms: methods, application, and perspective. Applied Microbiology Biotechnology, 93 (3) , pp.:993-1003.

Sugio, A. et al. (2015) 'Plant-insect interactions under bacterial influence: Ecological implications and underlying mechanisms', Journal of Experimental Botany, 66(2), pp. 467–478. doi: 10.1093/jxb/eru435.

Suh, S. O. *et al.* (2003) 'Wood ingestion by passalid beetles in the presence of xylose-fermenting gut yeasts', *Molecular Ecology*, 12(11), pp. 3137–3145. doi: 10.1046/j.1365-294X.2003.01973.x.

Surana, N.K. and Kasper, D.L. 2014. Deciphering the tête-à-tête between the microbiota and the immune system. The Journal of Clinical Investigation, 124(10), pp.4197-4203.

Swanson, K. S. *et al.* (2011) 'Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals similarities with humans and mice', *ISME Journal*. Nature Publishing Group, 5(4), pp. 639–649. doi: 10.1038/ismej.2010.162.

Tabatabai, N and Forst, S. 1995. Molecular analysis of the two-component genes, ompR and envZ, in the symbiotic bacterium Xenorhabdus nematophilus. Molecular Microbiology, 17, pp. 643-652.

Talla, V. *et al.* (2017) 'Rapid Increase in Genome Size as a Consequence of Transposable Element Hyperactivity in Wood-White (Leptidea) Butterflies', *Genome Biology and Evolution*, 9(10), pp. 2491–2505. doi: 10.1093/gbe/evx163.

Tang, W. H. W. and Hazen, S. L. 2014. The contributory role of gut microbiota in cardiovascular disease. Journal of Clinical Investigation, 124 (10).4204-4211

Tang, X., Freitak, D., Vogel, H., Ping, L., Shao, Y., Cordero, E. A., … Boland, W. 2012. Complexity and variability of gut commensal microbiota in polyphagous lepidopteran larvae. PLoS ONE, 7(7), pp. 1-9.

Tartar, A., Wheeler, M.M., Zhou, X., Coy, M.R., Boucias, D.G., Scharf, M.E. 2009. Parallel metatranscriptome analyses of host and symbiont gene expression in the gut of the termite Reticulitermes flavipes. Biotechnology Biofuels 2:25. doi: 10.1186/1754-6834-2-25.

Tipton, L., Darcy, J. L. and Hynson, N. A. (2019) 'A developing symbiosis: Enabling cross-talk between ecologists and microbiome scientists', Frontiers in Microbiology, 10(FEB), pp. 1–10. doi: 10.3389/fmicb.2019.00292.

Topping, D. L., & Clifton, P. M. (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiological reviews, 81*(3), 1031–1064. https://doi.org/10.1152/physrev.2001.81.3.1031

Tran, S. L. and Ramarao, N. (2013) 'Bacillus cereus immune escape: A journey within macrophages', *FEMS Microbiology Letters*, 347(1), pp. 1–6. doi: 10.1111/1574-6968.12209.

Triant, D. A., Cinel, S. D. and Kawahara, A. Y. (2018) 'Lepidoptera genomes: current knowledge, gaps and future directions', *Current Opinion in Insect Science*. Elsevier Inc., 25, pp. 99–105. doi: 10.1016/j.cois.2017.12.004.

Van Der Hoeven, R., Betrabet, G. and Forst, S. (2008) 'Characterization of the gut bacterial community in Manduca sexta and effect of antibiotics on bacterial diversity and nematode reproduction', *FEMS Microbiology Letters*, 286(2), pp. 249–256. doi: 10.1111/j.1574-6968.2008.01277.x.

VanBraekel, J. *et al.* (2020) 'Targeting the 16s rRNA gene for bacterial identification in complex mixed samples: Comparative evaluation of second (illumina) and third (oxford nanopore technologies) generation sequencing technologies', *j*. Multidisciplinary Digital Publishing Institute (MDPI), 21(1). doi: 10.3390/ijms21010298.

Vasanthakumar, A. *et al.* (2008) 'Gut microbiota of an invasive Subcortical Beetle, Agrilus planipennis fairmaire, across various life stages', *Environmental Entomology*, 37(5), pp. 1344–1353. doi: 10.1603/0046-225X(2008)37[1344:GMOAIS]2.0.CO;2.

Voirol, L. R. P. et al. (2018) 'Bacterial symbionts in lepidoptera: Their diversity, transmission, and impact on the host', Frontiers in Microbiology, 9(MAR), pp. 1–14. doi: 10.3389/fmicb.2018.00556.

Wang, F. *et al.* (2018) 'Assessment of 16S rRNA gene primers for studying bacterial community structure and function of aging flue-cured tobaccos', *AMB Express*. Springer Berlin Heidelberg, 8(1), p. 182. doi: 10.1186/s13568-018-0713-1.

Wang, J. *et al.* (2019) 'Core gut bacteria analysis of healthy mice', *Frontiers in Microbiology*, 10(APR), pp. 1–14. doi: 10.3389/fmicb.2019.00887.

Wang, L. T. et al. (2007) 'Comparison of gyrB gene sequences, 16S rRNA gene sequences and DNA-DNA hybridization in the Bacillus subtilis group', International Journal of Systematic and Evolutionary Microbiology, 57(8), pp. 1846–1850. doi: 10.1099/ijs.0.64685-0.

Wang, Q. *et al.* (2006) 'A bacterial carbohydrate links innate and adaptive responses through Toll-like receptor 2', *Journal of Experimental Medicine*, 203(13), pp. 2853–2863. doi: 10.1084/jem.20062008.

Wang, Shao-Yan Xu, Zhi-Gang Ren, Liang Tao, Jian-Wen Jiang, Shu-Sen Zheng.2015. Application of metagenomics in the human gut microbiome. World Journal Gastroenteroly, 21(3), pp. 803-814.

Warnecke, F. *et al.* (2007) 'Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite', *Nature*. Nature Publishing Group, 450(7169), pp. 560–565.

Woo, P. C. Y. *et al.* (2008) 'Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories', *Clinical Microbiology and Infection*. Elsevier, 14(10), pp. 908–934. doi: 10.1111/j.1469-0691.2008.02070.x.

Woods, H. A. (1999) 'Patterns and Mechanisms of Growth of Fifth-Instar Manduca sexta Caterpillars Following Exposure to Low- or High-Protein Food during Early Instars', *Physiological and Biochemical Zoology*. The University of Chicago Press, 72(4), pp. 445–454. doi: 10.1086/316678.

Yadav, K. K. *et al.* (2016) 'Diversity of cultivable midgut microbiota at different stages of the Asian tiger mosquito, aedes albopictus from Tezpur, India', *PLoS ONE*, 11(12), pp. 1–16. doi: 10.1371/journal.pone.0167409.

Yamada, S., Okashi, E., Agata, N. and Ventkateswaran, K. 1999. Cloning and nucleotide sequence analysis of gyrB of Bacillus cereus, B. thuringiensis, B. mycoides and B. anthracis and their application to the detection of B. cereus in rice. Applied Environmental Microbiology, 65, pp. 1483–1490

Yang, B., Wang, Y. and Qian, P. Y. (2016) 'Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis', *BMC Bioinformatics*. BMC Bioinformatics, 17(1), pp. 1–8. doi: 10.1186/s12859-016-0992-y.

Yarza, P. et al. (2014) 'Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences', Nature Reviews Microbiology. Nature Publishing Group, 12(9), pp. 635–645. doi: 10.1038/nrmicro3330.

Yashiro, E., Spear, R.N. and McManus, P.S. 2011. Culture-dependent and culture-independent assessment of bacteria in the apple phyllosphere, Journal of Applied Microbiology, 110, pp.1284- 1296.

Yoon, S. et al. (2017) 'Introducing EzBioCloud : a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies', pp. 1613–1617. doi: 10.1099/ijsem.0.001755.

Yumoto, I. *et al.* (2005) 'Oceanobacillus oncorhynchi sp. nov., a halotolerant obligate alkaliphile isolated from the skin of a rainbow trout (Oncorhynchus mykiss), and emended description of the genus Oceanobacillus', *International Journal of Systematic and Evolutionary Microbiology*. Microbiology Society, 55(4), pp. 1521–1524. doi: https://doi.org/10.1099/ijs.0.63483-0.

Yun, J. H. *et al.* (2014) 'Insect gut bacterial diversity determined by environmental habitat, diet, developmental stage, and phylogeny of host', *Applied and Environmental Microbiology*, 80(17), pp. 5254–5264. doi: 10.1128/AEM.01226-14.

Zaki, H. *et al.* (2010) 'Integrity and Mortality During Experimental Colitis', 32(3), pp. 379–391. doi: 10.1016/j.immuni.2010.03.003.The.

Zientz, E. *et al.* (2006) 'Relevance of the endosymbiosis of Blochmannia floridanus and carpenter ants at different stages of the life cycle of the host', *Applied and Environmental Microbiology*, 72(9), pp. 6027–6033. doi: 10.1128/AEM.00933-06.